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(71) Applicant: CELTRIX PHARMACEUTICALS, INC. [US/US]; 3055 Patrick Henry Drive, Santa Clara, CA 95054-1815 (US).

(72) Inventors: MASCARENHAS, Desmond; 27233 Sherlock Road, Los Altos Hills, CA 94022 (US). OLSON, Pamela, S.; 18630 Barnhart Avenue, Cupertino, CA 95014 (US).

(74) Agents: PARK, Freddie, K. et al.; Morrison & Foerster L.L.P., 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

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(54) Title: CHROMOSOMAL EXPRESSION OF HETEROLOGOUS GENES IN BACTERIAL CELLS

(57) Abstract

The present invention provides compositions and methods for producing a heterologous protein of interest by inserting a copy of a gene encoding the heterologous protein of interest into the chromosome of a host cell, such as *E. coli*. A chromosomal transfer DNA (a circular, non-self-replicating DNA) is used to integrate the gene encoding the heterologous protein of interest into the host cell chromosome. The chromosomal transfer DNA comprises at least one selectable marker and may optionally include repeated DNA sequences flanking the selectable marker, facilitating chromosomal amplification of the integrated DNA. The gene encoding the protein of interest may be expressed after integration into the chromosome of the host cell; selection for chromosomal amplification may be performed prior to expression of the gene.

CHROMOSOMAL EXPRESSION OF HETEROLOGOUS GENES IN BACTERIAL CELLS

TECHNICAL FIELD

This invention is related to the field of expression of heterologous genes in bacteria.

BACKGROUND ART

Genetic engineering has made it possible to produce large amounts of heterologous proteins or polypeptides in bacterial cells by means of recombinant expression systems, especially by expression in such prokaryotes as *Escherichia coli (E. coli)*.

The expressed heterologous proteins may be of mammalian, other eukaryotic, viral, bacterial, cyanobacterial, archaebacterial, or synthetic origin.

Unlike native bacterial proteins, which can often be efficiently accumulated within a bacterial cell even when encoded by a single chromosomal gene copy, there are no published reports to date of heterologous proteins being successfully accumulated within bacterial cells to levels exceeding 0.1% of total cell protein when expressed from a single chromosomal gene location.

0.1% of total cell protein (150 micrograms protein per trillion bacterial cells) is chosen as a practical measure of successful accumulation of protein because it approximately defines the lower limits of (a) economically significant accumulation of a desired protein by contemporary recombinant bacterial production standards, and (b) visual detection of a protein band by Coomassie-stained polyacrylamide gel analysis of whole bacterial cell extracts.

The relatively poor performance of non-bacterial genes when expressed in bacterial cells, even when placed under the control of the strongest known bacterial promoters, has been generally attributed to poor translation of the non-bacterial mRNAs

Previous methods for achieving the integration of heterologous genes into the chromosome of a bacterial host include the use of phage lambda vectors. The phage DNA in circular form is inserted linearly into the bacterial chromosome by a single site specific recombination between a phage attachment site (attP), 240 bases long, and a bacterial attachment site (attB), only 25 bases long. The two sites have 15 bases in common. This site-specific recombination is catalyzed by a special integrase, specified by the phage gene INT (VIROLOGY pp. 56-57 (Lippincott, 2nd ed., R. Dulbecco and H. Ginsberg, eds., Philadelphia, PA, 1985).

Phage vectors which are INT- can be integrated into the chromosome in a normal fashion as long as integrase is supplied in trans, e.g., by an INT+ helper phage (see, e.g., Borck et al. (1976) Molec. Gen. Genet. 146:199-207).

Phage vectors which are both att- and INT- can likewise be integrated into the bacterial chromosome as double lysogens by using att+INT+ helper phage. Double lysogens are formed by linkage of the prophages at the bacterial attachment site and are integrated into the chromosome by general bacterial recombination between homologous sequences on the defective phage and on the helper phage (see e.g., Struhl et al. (1976) Proc. Natl. Acad. Sci. USA 73:1471-1475). Similarly, it is also possible to integrate non-replicating colE1 replicons into the genome of polA strains of E. coli by means of recombination between the host chromosome and homologous sequences carried by the plasmid vector (Greener and Hill (1980) J. Bacteriol. 144:312-321).

More recently, systems have been specifically designed for the integration of foreign genes into a bacterial host chromosome. For example, U.S. Patent No. 5,395,763 (Weinberg et al.) discloses a chromosomal expression vector for the expression of heterologous genes. This vector was created utilizing a multicopy number plasmid intermediate, into which the gene of interest is cloned, placing the gene in operable linkage with the bacteriophage middle promoter, Pm. This plasmid intermediate, which comprises a defective Mu genome (lacking the genes necessary for the formation of phage particles) is introduced into a packaging strain to produce infectious Mu particles, which are then used to introduce the vector into host cells and integrate the vector into the host cell genome. This vector system is amplifiable once integrated into the host cell genome, but the mechanism of amplification (replicative

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for production of heterologous proteins in bacterial host cells such as E. coli by integrating a chromosomal transfer DNA (a circular, non-self replicating DNA) into the chromosome of a host cell. The chromosomal transfer DNA comprises one or more copies of a gene encoding the heterologous protein of interest.

The present invention, therefore, provides a method for producing a heterologous protein of interest, comprising:

integrating a chromosomal transfer DNA into the chromosome of a host cell such that chromosomal amplification of the integrated DNA is facilitated, the chromosomal transfer DNA comprising at least one copy of a gene encoding a heterologous protein of interest and a selectable marker; and

expressing the gene encoding the heterologous protein of interest, wherein the gene was at no time operably linked to a promoter functional in the host cell in a multicopy number plasmid during the construction of the transfer DNA, and

wherein the heterologous protein of interest accumulates to a level of at least 0.1% of total cell protein.

The chromosomal transfer DNA may optionally comprise a promoter operably linked to the gene encoding the heterologous protein of interest, wherein the operable linkage is created by circularization of the chromosomal transfer DNA.

Optionally, the chromosomal transfer DNA may further comprise duplicate DNA flanking the selectable marker. The duplicate DNA may optionally comprise copies of the gene encoding the heterologous protein of interest operably linked to a promoter.

The methods for expression of heterologous proteins may optionally include the step of selecting for chromosomal amplification.

The invention also provides methods for producing a chromosomal transfer DNA, comprising ligating together fragments from a first and a second plasmid vector:

Figure 2 shows a chromosomal transfer DNA formed from the ligation of two DNA fragments. One of the fragments contains a fusion gene comprising sequences encoding E. coli DsbA, yeast ubiquitin (beginning with a Met), and human insulin-like growth factor I ("dsbA-ubi-IGF") (not beginning with a Met), as discussed in co-owned, co-pending U.S. patent application no. 08/100,744, filed August 2, 1993. The other DNA fragment contains a T7 promoter. Both the chromosomal transfer DNA and the bacterial chromosome contain a recombination site from phage lambda, att P. The chromosomal transfer DNA is transformed into E. coli strain B1384, which makes integrase (INT) under the control of the trp promoter (P-trp). Integrase catalyzes site-specific integration of the chromosomal transfer DNA into the bacterial chromosome at the att site. The trp promoter can be induced during transformation by adding 1 mM indole acrylic acid (IAA) to the medium. Cells with integrated chromosomal transfer DNA sequences are resistant to chloramphenicol (CAM-r, 10 Tg/ml).

Figure 3 shows a B1384 chromosomal integrant resulting from the process described in Figure 2. The integration can be confirmed by amplifying host chromosomal DNA by PCR with various primer sets (e.g., UBUF x IGFR, 1243 x T7REV, or TRPPF x 1239), digesting the amplified fragments with the appropriate restriction enzyme (SacII, HinCII, or BamHI, respectively), and sizing the products by gel electrophoresis).

Figure 4 shows a Western blot of whole cell lysates of chloramphenicol resistant W3110DE3 transductants. Also included are protein size markers (far left lane) and IGF fusion protein (control).

Figure 5 shows a Western blot of whole cell lysates of kanamycin resistant transductants.

Figures 6-9 show diagrammatically the general strategy for construction of chromosomal transfer DNA's. Figure 6 shows a chromosomal transfer DNA comprises a single copy of the gene encoding the heterologous protein of interest and two copies of a second gene which flank the selectable markers, facilitating chromosomal amplification after integration of the chromosomal transfer DNA. Figure 7 shows the "double cassette" system utilized for expression of heterologous proteins in Examples 2 through 6 and Example 8. This embodiment of the chromosomal transfer

express a DsbA::ubiquitin::IGF-I fusion protein (left arrow), which is easily visible. Surprising, this high level of expression is seen in c49222 and c49258#46, which were not amplified (i.e. there was no selection for chromosomal amplification of the integrated DNA). c57264#5 and c57264#28 express a DsbA::3C::IGF-I fusion protein while c57265#44 and c57265#54 express a DsbA::2A::IGF-I fusion protein. Again, the expressed fusion protein is easily visible. Densitometric analysis of this gel indicates that all of the isolates accumulate protein in excess 19% of total cell protein (average protein accumulation is 25.7% of total cell protein).

Figure 24 shows a Southern blot of chromosomal DNA isolated from c49222, c49258#46, c53063, c57264#5, c57264#28, c57265#44, and c57265#54. The blot was probed with a DNA fragment encoding ubiquitin fused to IGF-I. The higher molecular weight band in each lane represents a single copy of the integrated IGF-I fusion protein gene in each isolate. The lower molecular weight band also represents the integrated IGF-I fusion protein gene, but this fragment can be amplified by chromosomal amplification. Isolates c53063, c57264#5, c57264#28, c57265#44, and c57265#54 have clearly been amplified, showing about 3 to 5 fold amplification.

Figure 25 shows coomassie blue-stained SDS-PAGE gels showing protein accumulation in isolates carrying integrated genes encoding IGFBP-3 fusion proteins. A) shows protein accumulation in an isolate expressing a DsbA::2A::IGFBP-3 fusion protein. The right lane shows protein expression after induction of T7 RNA polymerase by addition of IPTG to the culture medium. B) shows protein accumulation in an isolate expressing a DsbA::3C::IGFBP-3 fusion protein. As in Figure 23, the bands representing the fusion protein are easily visible. Densitometric scanning of these gels found that the accumulated protein represented 22.6% in Panel A, and the two isolates in Panel B accumulated 33.% and 28.2% of total cell protein (left to right, respectively).

Figure 26 shows a coomassie blue-stained SDS-PAGE gel showing protein accumulation from host cells expressing a gene encoding DsbA::ubiquitin::TGF-β2. M indicates molecular weight markers and C indicates a positive control. The two Plasmid lanes (Lanes 1 and 2) are used as a standard to compare protein accumulation from multicopy number plasmid vectors to protein accumulation from genes integrated into the chromosome. Lanes 3 and 4 are whole cell

The present invention employs "chromosomal transfer DNA" which may be used to simply, efficiently, and reliably insert a copy of a heterologous gene into the chromosome of a host cell, e.g., E. coli. A chromosomal transfer DNA is a circular DNA comprising one or more copies of a gene encoding a heterologous protein of interest, a selectable marker (e.g., an antibiotic resistance gene), a recombination site (e.g., a site-specific recombination site such as lambda attP or attB or a DNA sequence homologous to a segment on the host cell chromosome), and means for facilitating the amplification of the chromosomal transfer DNA following recombination into the host cell chromosome, and lacking an origin of replication or autonomously replicating sequence (ARS). The chromosomal transfer DNA is therefore incapable of replicating independently when introduced in to the host cell. The chromosomal transfer DNA may optionally carry a promoter operably linked to the gene of interest.

When a chromosomal transfer DNA carrying a site-specific recombination site is introduced into a host cell having a chromosome which contains a second, similar recombination site (e.g., another attP or attB site), expression in the host cell of an enzyme which is capable of catalyzing the site-specific recombination of the recombination sites (e.g., integrase) results in the integration of the vector into the host cell chromosome at the recombination site. This site-specific recombination process is much more efficient than general recombination systems acting on homologous vector and host chromosomal sequences and results in integrated sequences having greater stability, particularly when integrase synthesis can be controlled. Integrase may also be provided by a plasmid or other DNA molecule transiently or stably present in the host cell at the time when the chromosomal transfer DNA is introduced.

It will be apparent to one skilled in the art that there are a variety of methods other than the preferred method utilizing attP, attB, and INT which may be used to integrate a chromosomal transfer DNA into the chromosome of a host cell. For example, non-replicating colE1 replicons, transposable elements, or even naked DNA carrying sequences homologous to sequences found on the host chromosome may be used to insert the chromosomal transfer DNA into the host chromosome. The multicopy colicin plasmids ColE1, CloDF13, ColK, and ColA all comprise site-specific recombination systems including a cis- and trans-acting element. For use in the present

circular DNA, which would result in circularization of the insert. Preferably, circularization is accomplished by ligation of one or more DNA fragments.

Alternatively, high level expression of less toxic gene products can be accomplished by multiple integrations or by selection for amplification of integrated genes.

Recombinant DNA Methods and Reagents

General techniques for nucleic acid manipulation useful for the practice of the claimed invention are described generally, for example, in Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, Vols. 1-3 (Cold Spring Harbor Laboratory Press, 2 ed., (1989); or F. Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Green Publishing and Wiley-Interscience: New York, 1987) and periodic updates. Reagents useful in nucleic acid manipulation, such as restriction enzymes, T7 RNA polymerase, DNA ligases and so on are commercially available from such vendors as New England BioLabs, Boerhinger Mannheim, Amersham, Promega Biotec, U.S. Biochemicals, and New England Nuclear.

Definitions

"Foreign" or "heterologous" or "non-bacterial;" "native" or "homologous" A "foreign or "heterologous" polypeptide is a polypeptide which is not normally found in a host cell of a particular species. The nucleic acid encoding such a polypeptide is also referred to as "foreign" or "heterologous." For example, insulin-like growth factor (IGF), insulin-like growth factor binding protein (IGFBP), and transforming growth factor-beta (TGF-β) are native to mammalian cells and human rhinovirus 3C protease is native to viruses and virally-infected mammalian cells, but these proteins are foreign or heterologous to E. coli. A "non-bacterial protein" is a protein or polypeptide which is not naturally found in a bacterial cell. Non-bacterial proteins include viral and eukaryotic proteins. Non-bacterial, foreign, or heterologous proteins may also be fusions between non-bacterial, foreign, or heterologous proteins and other proteins or polypeptides. For the embodiments encompassed by this invention, both "heterologous protein" and "non-bacterial protein" may be expressed. As disclosed

multicopy number plasmids or in vitro amplification such as the polymerase chain reaction (PCR).

Probes and primers

Nucleic acid probes and primers are isolated nucleic acids, generally single stranded, and, especially in the case of probes, are typically attached to a label or reporter molecule. Probes are used, for example, to identify the presence of a hybridizing nucleic acid sequence in a tissue or other sample or a cDNA or genomic clone in a library. Primers are used, for example, for amplification of nucleic acid sequences, e.g., by the polymerase chain reaction (PCR). The preparation and use of probes and primers is described, e.g., in Sambrook et al., supra or Ausubel et al. supra.

Chemical synthesis of nucleic acids

Nucleic acids, especially short nucleic acids such as amplification primers, may be produced by chemical synthesis, e.g., by the phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862 or the triester method according to Matteucci et al. (1981) J. Amer. Chem. Soc. 103:3185, and may be performed on automated oligonucleotide synthesizers. A double-stranded fragment may be obtained from the single-stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strands together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Features of chromosomal transfer DNA and of plasmids used in their construction

Chromosomal transfer DNA comprises a DNA fragment encoding a selectable marker and a sequence encoding a desired heterologous polypeptide. Optionally, a chromosomal transfer DNA may also comprise, in an operable linkage to the sequence encoding the desired heterologous polypeptide, transcription and translation initiation regulatory sequences and expression control sequences, which may include a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, and mRNA stabilizing sequences, as well as any necessary

ENHANCERS AND EUKARYOTIC GENE EXPRESSION (Cold Spring Harbor Press, New York, 1983).

It is preferable that the promoter driving expression of the heterologous gene when integrated in the chromosome of the host is controllable.

Chromosomal transfer DNAs and plasmids employed in their construction generally comprise a selectable marker, a gene encoding a protein necessary for the survival or growth of a host cell transformed with the chromosomal transfer DNA or plasmid. Typical selectable markers (a) confer resistance to antibiotics or other toxic substances, e.g. ampicillin, neomycin, methotrexate, etc.; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for Bacilli. The choice of the proper selectable marker will depend on the host cell.

The chromosome transfer DNAs of the present invention may contain a site-specific recombination site, such as the phage lambda attP site. When transformed into a bacterial host strain (such as E. coli B1384) which makes the enzyme integrase, integrase recognizes the attP site on the chromosomal transfer DNA and catalyses its recombination with an att site (integrase can catalyze a recombination between two attP and attB or two attP sites). Bacterial host cells bearing the integrated DNA are selected for on the basis of a selectable marker carried on the integrated DNA.

Thus, integration utilizing site-specific recombination generally involves expression of an enzyme such as integrase which can catalyze site-specific recombination and the presence of a site recognized by the enzyme on both the chromosomal transfer DNA and the bacterial chromosome. Other site-specific recombination systems characterized by an "integrase" or similar enzyme and sites specifically recognized by the "integrase" could be used as well.

High level expression of a foreign gene integrated into the chromosome of a host cell in multiple copies is also possible, e.g., by incorporating multiple att sites in the host cell chromosome and introducing multiple chromosomal transfer DNAs into the host cell. Additionally or alternatively, host cells containing multiple copies of the integrated DNA may be obtained by selecting for chromosomal amplification. Chromosomal amplification is facilitated when the selectable marker is flanked by

There are several methods by which one may construct a chromosomal transfer DNA using two or more DNA sources. In one preferred embodiment, shown in Figure 7, also uses two DNA sources. In this embodiment, each of the two DNA sources carries a copy of the gene encoding the heterologous protein of interest and the promoter, but the gene encoding the heterologous protein of interest and promoter are not operably linked on either DNA source. As with the previously described embodiment, other necessary sequences may be carried by either DNA source (alternatively the other necessary sequences may be provided by one or more accessory DNA sources). The two DNA sources are cleaved, then joined to each other, forming a circular chromosomal transfer DNA which has two copies of the foreign gene, each operably linked to a copy of a promoter. The promoter from the first DNA source is operably linked to the gene encoding the heterologous protein of interest from the second DNA source, and the promoter from the second DNA source is operably linked to the gene encoding the heterologous protein of interest from the first DNA source.

Chromosomal transfer DNAs may also be designed without promoters (Figures 8 and 9). These promoter-less chromosomal transfer DNAs are integrated into target sites on the bacterial chromosome which place the gene encoding the heterologous protein of interest into an operable linkage with a promoter on the host cell chromosome. The chromosomal transfer DNA of this embodiment includes a copy of a gene encoding a heterologous protein of interest linked in-frame to a segment of target-site DNA segment homologous to DNA on the host cell chromosome and a selectable marker. This target site DNA sequence will typically be the 5' end of a gene located on the bacterial chromosome downstream from a promoter. Integration of the chromosomal transfer DNA into the host cell chromosome will place the gene encoding the heterologous protein of interest into operable linkage with a bacterial promoter. The target sequence on the host cell chromosome may be a naturally occurring sequence or may be a site which is introduced into the chromosome of the host cell. A target may be introduced into the chromosome of a host cell utilizing a DNA sequence homologous to a segment of the host cell chromosome, as described above for integration of the chromosomal transfer DNA. A target site may also be introduced using site-specific recombination, such as the attB/attP/INT system described above. A target site sequence

Integrated DNA which contains or is flanked by duplicate DNA sequences of 25 or more base pairs will form chromosomal duplications (Normark et al. (1977) J. Bacteriol. 132:912-922; Edland et al. (1979) Mol. Gen. Genet. 173:115-125; Tlsty et al. (1984) Cell 37:217-224; Stern et al. (1984) Cell 37:1015-1026). Selection for duplications (amplification) is greatly facilitated if the duplicate DNA contains a selectable marker, such as an antibiotic resistance gene or a gene which complements a host cell deficiency. Preferably the integrated DNA includes two selectable markers; a first selectable marker which is operable at low copy number and is used to select for integrants, and an second selectable marker which requires high copy number and is used to select for host cells which have amplified the integrated DNA. Amplification may also be accomplished by replicative transposition, in the case where the chromosomal transfer DNA contains the appropriate transposon sequences or the chromosomal transfer DNA is integrated into a transposon. Preferably, amplification is accomplished by selection for chromosomal duplications.

chromosomal transfer DNA and in the chromosome of B1384 and catalyses their recombination, leading to the site-specific integration of the chromosomal transfer DNA into the bacterial chromosome at the att P site (Weisberg et al. Comprehensive Virology, vol. 8, pp. 197-258 (Plenum, Fraenckel-Conrat and Wagner, eds., New York, NY, 1977). Bacterial host cells bearing the integrated DNA are selected for on the basis of their resistance to chloramphenicol.

Chloramphenicol-resistant chromosomal integrants were tested as summarized in Figure 3. The presence of the integrated chromosomal transfer DNA was confirmed by amplifying host chromosomal DNA by PCR with the following primer sets (e.g., UBUF x IGFR, 1243 x T7REV, or TRPPF x 1239)

IGFR: 5' ... CCC ATC GAT GCA TTA AGC GGA TTT AGC CGG TTT CAG...3'

#1239: 5'...GCC TGA CTG CGT TAG CAA TTT AAC TGT GAT...3'

#1243: 5;...CTG GGC TGC TTC CTA ATG CAG GAG TCG CAT...3'

#1227: 5'...TAA TAC GAC TCA CTA TAG GGA GA...3'

TRPPF: 5'...GAT CTG TTG ACA ATT AAT CAT CGA ACT AGT TAA CTA GTA CGC AAG TT...3'

T7REV: 5'...TGC TAG TTA TTG CTC AGC GG...3'

CYCF1: 5'...CAG GAT CCG ATC GTG GAG GAT GAT TAA ATG GCG AAA GGG GAC CCG CAC...3'

CYCR1: 5'...CAG GAA GCT TAC GGC AGG ACT TTA GCG GAA AG...3'

UBUF: 5'...GGG GCC GCG GTG GCA TGC AGA TTT TCG TCA AGA CTT TGA...3'

The amplified fragments were digested with the appropriate restriction enzyme (SacII, HinCII, or BamHI, respectively). The products were sized by agarose gel electrophoresis. Presence of the integrated sequences was demonstrated by amplification of:

- The results obtained were identical in all six independent cases: the chromosomal transfer DNA was transferred with high efficiency to a new location on the bacterial chromosome, the att sites flanking the prophage in W3110DE3. This was confirmed by
  - chloramphenicol resistance;
  - no plasmid DNA in DNA minipreps;
  - i21 immunity (DE3 lysogen; phage lysates were plated on bacterial lawns by standard techniques);
  - gal^+ (i.e. growth on galactose minimal plates);
  - expression of IGF protein under lac control (expression and analysis carried out as described in Example 1 or co-owned, co-pending U.S. patent application Serial No. 08/101,506, filed August 2, 1993).

Chromosomal DNA from the six strains ("integrants") was digested to completion with BglII and NcoI and a Southern blot of the digested DNA was probed with a labeled 0.6 kb DsbA DNA probe which covers the entire gene sequence coding for mature DsbA (Bardwell et al. (1991) Cell 67:581-589; see also Kamitani et al. (1992) EMBO J 11:57-62). Each of the six integrants contained insertions; the blots demonstrated the existence of several double insertions, one single insertion, and one (isolate WB3-6) apparently duplicate double (i.e. triple) insertion.

The six integrants were tested for expression of the IGF fusion protein after induction with isopropyl-J-thiogalactopyranoside (IPTG). Cells were induced with IPTG for two hours and whole cell extracts for the induced integrants, as well as size markers and an IGF fusion protein control, were separated by 12% SDS-PAGE, Western blotted, and reacted with polyclonal anti-IGF sera (see Example 1 of co-owned, co-pending U.S. patent application Serial No. 08/101,506, filed August 2, 1993) (Figure 4). Isolate WB3-6 (Figure 4, lane 6) showed the highest levels of expression of the IGF fusion protein. An induced band of the same size was also seen on Coomassie blue-stained gels.

A different binary system was used to generate a chromosomal transfer DNA carrying a kanamycin resistance marker. The plasmids used, pDM25424 and pDM25427, are described in the figures. The configuration and location of the insert

The double cassette binary system utilizes two plasmids, pDM25470 and pDM25465, as shown in Figures 7 and 14. pDM25425 is a pUC19 derivative carrying a copies of attP, the T7 promoter, and a copy of the rrnt1t2 terminator, from which a 1.6 kb fragment was deleted by BglII/BamHI digestion. A terminator and a sequence encoding DsbA (a 1.5 kb NcoI(fill)/NsiI fragment from pDM25463) was added ligated to EcoRI(fill)/NsiI-digested pDM25459 to form pDM25470 (one of the double cassette binaries). The other double cassette plasmid, pDM25465, carries two copies of a terminator, a kanamycin resistance gene, and the cyclophilin gene (the use of the cyclophilin gene to aid in protein production is described in co-owned, co-pending U.S. patent application Serial Number 08/101,506, incorporated herein by reference in its entirety). The cyclophilin gene was cloned from pER15951 (HinDIII(fill)/XbaI, 0.6 kb fragment) into pDM25424 (BamHI(fill)/XbaI, 5.2 kb fragment; a pUC19 backbone carrying two copies of a terminator and a kanamycin resistance gene). The kanamycin resistance gene in pDM25430 (derived from pDM25424) was insufficiently effective, so it was replaced with a kanamycin resistance gene from pLG339hly (PvuII/EcoRI digest), creating plasmid pDM25443. The T7 promoter was cloned into pDM25443 by annealing oligos T7F and T7R and ligating them the EcoRI-digested pDM25443, creating pDM25465.

Two sets of oligonucleotides were synthesized (1, 2, 1R, 2R and 3, 4, 3R, 4R), phosphorylated, denatured, and annealed. The annealing product of 1, 2, 1R, and 2R, which encodes ubiquitin, was ligated into pUC18 (SphI-BamHI digest). The annealing product of 3, 4, 3R, and 4R, which encodes IGF-I, was ligated into pUC18 (EcoRI-BamHI digest). The resulting plasmids were transformed into JM109 and the transformed host cells were selected on ampicillin plates. Transformants were analyzed for the presence of the ubiquitin and IGF-I sequences, then sequenced to identify correctly formed constructs. One isolate from each was selected, and designated pPO39354 and pPO39334, respectively.

1

5'-CAG ATT TTC GTC AAG ACT TTG ACC GGT AAA ACC ATA ACA TTG GAA GTT GAA CCT TCC GAT ACC ATC GAG AAC GTT AAG GCG AAA ATT CAA GAC AAG GAA GGT ATC CCT CCA GAT CA-3'

The fusion gene from pPO39358 was ligated into the double-cassette binary parent vectors pDM25470 and pDM25465 to create pPO39377 and pPO41623, respectively. EcoRI-XbaI fragments of pPO39377 and pPO41623 were ligated to form the chromosomal transfer DNA (Figure 17).

The chromosomal transfer DNA was transformed into E. coli strain B1384, which contains an attP site as well as a sequence, under the control of the trp promoter, encoding the enzyme integrase (INT). Indole acrylic acid (1 mM) was added to induce the expression of INT and resulted in the integration of transduced chromosomal transfer DNAs. Cells were tested for chromosomal transfer DNA integration by:

Blue/yellow screening Cells were tested for integrated DNA by blue/yellow screening with AmpScreen (BRL). Colonies with a blue phenotype were further screened, yellow colonies were discarded.

PCR Cells were tested for properly integrated DNA by amplification of host cell chromosomal DNA using primer pairs:

T7F1 5'-AAT TGT CGA CAT TAA TAC GAC TCA CTA TAG GGA GAC CAC AAC GGT TTC CCT GAA TTG TCG ACA TTA ATA CGA CTC ACT ATA GGG AGA CCA CAA CGG TTT CCC TG-3'

IGFREV 5'-CCC ATC GAT GCA TTA AGC GGA TTT AGC CGG TTT CAG-3'

which confirm the presence of the complete fusion gene with its promoter and

T7REV 5'-TGC TAG TTA TTG CTC AGC GG-3'

TRPBR2 5'-AAG GGC TTC ATC ATC GGT AAT AGA CA-3'

which confirm the integration of the chromosomal transfer DNA into the att site of B1384.

Production of protein from integrated genes requires T7 RNA polymerase activity, which is lacking in B1384. To test protein production from the integrated gene, P1 lysates were made using a B1384 integrant. The lysates were then transduced into E. coli strain W3110DE3 (as described in Example 1), which is Gal^+ and carries a copy of the T7 RNA polymerase gene under the control of the lac promoter. Transductants were

GTT AAG GCG AAA ATT CAA GAC AAG GAA GGT ATC CCT CCA GAT CA-3'

1224 5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'

A P1 lysate was then made from an isolate which was kan^r/cam^r and integrated into the lac region. This P1 was used to transduce W3110DE3. Transductants were selected for kanamycin and chloramphenicol resistance by growth on selective media. Kan^r/cam^r isolates were tested for T7 RNA polymerase activity by streaking against phage 4107 as described above. Two isolates positive for T7 RNA polymerase activity, designated c49258#46 and c49258#50, were tested for protein accumulation by induction with IPTG for two hours. Whole cell lysates were analyzed by SDS-PAGE using 12.5% acrylamide gels. DsbA::ubiquitin::IGF-I fusion protein accumulated to 19.6% of total cell protein in c49258#46, as measured by densitometry of an SDS-PAGE gel.

Southern blot analysis of chromosomal DNA from c49222 and c49258#46 was performed to check the copy number of the integrated DNA. Chromosomal DNA from c49222 and c49258#46 was isolated, digested with restriction endonucleases, transferred to Hybond-N (Amersham), and probed with the a DNA fragment encoding the ubiquitin and IGF-I portions of the fusion protein. Analysis of the Southern blot showed that there were approximately two copies each of the DsbA::ubiquitin::IGF-I gene integrated into the chromosomes c49222 and c49258#46 (Figure 24), i.e. a single copy of the integrated DNA). This result was surprising and unexpected in view of the levels of accumulation of DsbA::ubiquitin::IGF-I protein shown by SDS-PAGE (22.3% and 19.6% of total cell protein, respectively). Ordinarily, it is expected that such high levels of protein accumulation can only be accomplished by expression of heterologous genes carried by high copy number plasmids.

DsbA::ubiquitin::IGF-I was also produced by integrating a chromosomal transfer DNA carrying a gene for tetracycline resistance in addition to the gene for kanamycin resistance. P1 lysates prepared from a B1384 integrant were used to transduce W3110DE3 to kanamycin resistance (see Example 1). Kan^r isolates were checked for properly integrated DNA using primer pairs T7F1 x IGFREV and ATT3 x T7RNAP1 as described above. Isolates were also tested for T7 RNA polymerase activity

DsbA::ubiquitin::IGF-I fusion protein substrate at 37° C for one hour. Cleavage was monitored by SDS-PAGE. All isolates (WBD311, 312, 313, 314, 331, and 332) showed good levels of enzyme activity (i.e. complete cleavage of the substrate under assay conditions).

Example 4

Expression of an insulin-like growth factor binding protein-3 (IGFBP-3) fusion protein

A chromosomal transfer DNA carrying a fusion protein comprising DsbA, a linker including a human rhinovirus 2A protease site, and IGFBP-3 (DsbA::2A::IGFBP-3) was created using the double cassette method. Construction of the fusion gene and chromosomal transfer DNA are shown in Figure 18. DsbA was from pDM46905, the 2A protease site was created by annealing primers V2ATA and V2ATB, and IGFBP-3 was PCR amplified from pYZ42580 using primers BP3RZ and NBP3F.

The IGFBP-3 gene used to create the DsbA::2A::IGFBP-3 fusion was created by annealing and ligating a number of synthetic oligonucleotides, which, when fully assembled, code for IGFBP-3 protein. The oligonucleotides were assembled in three segments; 5', 3', and middle. Oligonucleotides

F1-1 5'-AGC TTG GTG CTT CTT CTG CTG GTC TTG GAC CAG TTG TTC GTT GTG AAC CAT GTG ATG CAC GAG CTT TAG CTC AAT GTG CTC CAC CAC CAG CTG TT-3',

F1-2 5'-TGT GCT GAA TTA GTT CGA GAA CCA GGT TGT GGT TGT TGT TTA ACT TGT GCT TTA TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT TGC GG-3',

F1-3 5'-TAG TGG TTT GCG TTG TCA ACC AAG CCC AGA TGA AGC TAG GCC TTT ACA AGC ATT ATT AGA TGG TCG AGG TCT GTG TGT TAA TGC GTC CGC TGT TTC TCG ATT GCG CGC G-3',

C1-1 5'-TCG ACG CGC GCA ATC GAG AAA CAG CGG ACG CAT TAA CAC ACA GAC CTC GAC CAT CTA ATA ATG CTT GTA AAG GCC TAG CTT CAT CTG GGC TTG GTT G-3',

C1-2 5'-ACA ACG CAA ACC ACT ACC GCA ACG TTC AGT ATA AAT ACC ACA TGG TTG ACC TTC AGA TAA AGC ACA AGT TAA ACA ACA ACC ACA ACC TGG TTC TC-3',

MF2 5'-GCG AAG AGG ATC GTT CTG CGG GTT CCG TTG AAT CTC CAA GTG TGA GTT CTA CCC ATC GAG TTA GCG ACC CGA AA-3',

MF3 5'-TTT CAT CCG TTG CAC TCT AAA ATC ATT ATT ATT AAA AAG GGT CAC GCA AAG GAT TCT CAA CGT TAT AAG GT-3',

MF4 5'-GGA TTA TGA AAG CCA ATC TAC CGA CAC TCA AAA TTT TAG TAG TGA AAG TAA ACG TGA AAC CGA GTA CGG CCC GTG-3',

MB1 5'-TCG ACA CGG GCC GTA CTC GGT TTC ACG TTT ACT TTC ACT ACT AA-3',

MB2 5'-AAT TTT GAG TGT CGG TAG ATT GGC TTT CAT AAT CCA CCT TAT AAC GTT GAG AAT CCT TTG CGT GAC CCT TTT T-3',

MB3 5'-AAT AAT AAT GAT TTT AGA GTG CAA CGG ATG AAA TTT CGG GTC GCT AAC TCG ATG GGT AGA ACT CAC ACT TGG AGA TT-3',

and

MB4 5'-CAA CGG AAC CCG CAG AAC GAT CCT CTT CGC TTT CGG AGG CGT TAC CCG GTG CCG GTG GGG CAG GTA ATA AAT AAG-3',

digesting the ligated DNA with BssHII and SalI, end filling with Klenow then cloning into Klenow-filled, XbaI-digested pUC18.

PCR amplification of a segment of pYZ37490 was used to add a SacII site and repair a cloning artifact. Primer pairs

pF1 5'-GGT TGT TGT TTA ACT TGT GCT TTA TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT TGC GGT AGT GGT TTG CGT TGT CAA CCA AGC CCA GAT GAA GCT AGG-3'

1233 5'-AGC GGA TAA CAA TTT CAC ACA GGA-3'

and

pR1 5'-TAA AGC ACA AGT TAA ACA ACA ACC ACA ACC TGG TTC TCG AAC TAA TTC AGC ACA AAC AGC TGG TGG TGG AGC ACA TTG AGC TAA AGC TCG TGC ATC ACA TGG T-3'
1224 5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'

This repaired a cloning defect and added a SalI site. Two DNA fragments were amplified from pYZ42529 using primer pairs 715F1' x 1233 and 715R' x 1224. These two fragments were mixed and PCR amplified into a single DNA fragment using 1233 x 1224. This single fragment was digested with BssHI and SalI, then ligated into a BssHI-SalI digest of pYZ42529, creating pYZ50559.

pYZ42580, the donor construct for the IGFBP-3 gene, was created by ligation of EcoRI-SacII fragments from pYZ50559 and pDM25497.

The chromosomal transfer DNA carrying the DsbA::2A::IGFBP-3 fusion gene were transfected into E. coli strain B1384, which was grown in the presence of 100 1M IAA to induce the expression of INT and the integration of the chromosomal transfer DNA. Integrants were selected with kanamycin. All isolates were also ampicillin sensitive.

Isolates were further characterized by diagnostic PCR amplification of the host cell chromosome. PCR amplification with primer pairs

1227 5'-TAA TAC GAC TCA CTA TAG GGA GA-3'
BP3-607 5'-GGG ATA TGA ACG CCA CGC GGG GAT AA-3',

INT107 5'-GCG GAG AAA CCA TAA TTG CAT CTA CTC-3'
BP3-559 5'-CGT GAA ACC GAG TAC GGC CCG TGT C-3,'

and

T7REV 5'-TGC TAG TTA TTG CTC AGC GG-3'
TRPBR2 5'-AAG GGC TTC ATC ATC GGT AAT AGA CA-3'

confirmed the proper integration of the intact chromosomal transfer DNA into the chromosome at the att site.

P1 lysates were prepared from a single isolate and used to transduce W3110DE3 to kanamycin resistance (as described in Example 1). Kanamycin resistant isolates were assayed for T7 RNA polymerase activity by streaking against phage 4107, as described in Example 2. Isolates with T7 RNA polymerase activity were then tested for expression of the fusion gene by induction with IPTG, followed by analysis of protein expression by SDS-PAGE of whole cell lysates on 12.5% polyacrylamide gels. Densitometric analysis of whole cell lysates indicated that the DsbA::2A::IGFBP-3 fusion protein accumulated to a level of 22.6% (Figure 25 A).

IPTG. Protein expression was assayed by SDS-PAGE of whole cell lysates. Densitometric scanning of a SDS-PAGE gel showed that the two isolates expressing DsbA::3C::IGF-I fusion protein accumulated the fusion protein to 20% and 20.1% of total cell protein and the two isolates expressing DsbA::2A::IGF-I accumulated the fusion protein to 25.7% and 38% of total cell protein.

Example 6

Chromosomal expression of TGF-β2 using the double cassette binary system

A chromosomal transfer DNA encoding a fusion protein comprising DsbA, ubiquitin, and human TGF-J2 (DsbA::ubiquitin::TGF-β2) was created using the double cassette method. Construction of the fusion gene and chromosomal transfer DNA are shown in Figure 21. DsbA::ubiquitin was from pDM25497, and TGF-J2 was PCR amplified from pPC-21 (Madisen et. al. (1988) DNA 7:1-8) using primers

UBTGFJ2F 5'-GGG GCC GCG GTG GTG CTT TGG ATG CGG CCT ATT GCT TTA GA-3'
and

TGFJ2R 5'-GGG GAA TTC TTA GCT GCA TTT GCA AGA CTT TAC A-3'.

pDM25497 was digested with SacII-EcoRI and the 4.3 kb fragment containing pUC18 and DsbA::ubiquitin sequences was isolated. The 0.35 kb PCR product resulting from the amplification of pPC-21 encoding the last 112 amino acids of human TGF-J2 was purified and digested with SacII-EcoRI. These two fragments were ligated to create pDP26, a pUC18 derivative containing a DsbA::ubiquitin::TGF-J2 fusion gene. pDP26 was the donor construct for assembly of the binary plasmids used to make the chromosomal transfer DNA.

The fusion gene from pDP26 was ligated into the double-cassette binary vectors pDM25470 and pDM25465 to create pC9DP and pA6DP, respectively. Briefly, pDM 25470 was digested with BamHI-SmaI and the 4.2 kb fragment was isolated. pDP26 was digested with EcoRI, blunt ended with the Klenow fragment of DNA polymerase, and then digested with BamHI. The 1.1 kb fragment from this digest was isolated. The two fragments described above were ligated to create pC9DP.

analysis of protein expression by SDS-PAGE of whole cell lysates on 10% polyacrylamide gels (Figure 26). Protein accumulation in chromosomal integrants was comparable to the levels seen in host cells containing a multicopy number plasmid utilizing the same T7 promoter linked to the a copy of the gene encoding the DsbA::ubiquitin::TGF-β2 fusion protein. Densitometric analysis showed that protein accumulation in chromosomal integrants was as high as 36.7% of total cell protein.

Example 7

Expression of a heterologous protein using a promoter-less CTD

This example shows the use of a chromosomal transfer DNA which does not carry a promoter. The chromosomal transfer DNA carries a segment of DNA homologous to a bacterial gene (in this example, lacZ or DsbA) linked in-frame to a DNA sequence encoding a heterologous protein of interest (in this case the DsbA::3C::IGF-I fusion protein of Example 5), as well as selectable marker genes. The homologous DNA encodes the 5' region of the bacterial gene. The chromosomal transfer DNA is introduced into the host cell, where it integrates into the homologous gene on the chromosome of the host cell, forming an operable linkage between the homologous gene's promoter and the DNA sequence encoding the heterologous protein of interest. Integrants are selected for using the selectable markers carried on the chromosomal transfer DNA. The heterologous protein of interest is expressed through the homologous gene's promoter (Figure 8).

The DNA encoding the DsbA::3C::IGF-I fusion protein is constructed as described in Example 5. This fusion gene is then placed in frame to a DNA segment encoding the first 100 amino-terminal amino acids of the lacZ gene, forming a lacZ/DsbA::3C::IGF-I gene. The cyclophilin, kanamycin resistance, and tetracycline resistance genes utilized in Example 5 are also cloned into the plasmid carrying the lacZ/DsbA::3C::IGF-I gene. This plasmid is then cleaved with restriction endonucleases to remove the plasmid origin of replication, the ampicillin resistance gene and other non-essential sequences, then re-ligated to form a circular chromosomal transfer DNA. The chromosomal transfer DNA is transformed into E. coli host cells and the transformed host cells are grown on media containing kanamycin (10 μg/ml).

PCR amplification of host cell chromosomal DNA using primer pairs T7F1 x IGFREV confirms the integration of the intact chromosomal transfer DNA. Integrants are checked for T7 RNA polymerase activity by streaking against phage 4107, as described in Example 2. Amplification of the integrated DNA is selected for by growth of T7 RNA polymerase-positive isolates on kanamycin, chloramphenicol, and tetracycline. Resistant isolates are assayed for protein expression by induction with IPTG. Protein expression is assayed by SDS-PAGE.

Example 8

Expression of a DsbA::3C::IGFBP-3 fusion protein using the double cassette system

A gene encoding DsbA::3C::IGFBP-3 fusion protein was expressed using the double cassette binary system shown in Figure 7. The DsbA sequence was originally isolated by PCR amplification of the DsbA gene from the E. coli chromosome; plasmid pDM25454 was used as the source of the DsbA sequence for this fusion gene. The site for 3C protease was created by synthesizing two oligonucleotides,

RV3CTA 5'-CCCGATTCTCTGGAAGTTCTGTTCCAA-3'

and

RV3CTB 5'-TTGGAACAGAACTTCCAGAGAATCGGGCATG-3',

which were annealed to form a double stranded DNA fragment encoding a 3C protease cleavage site. The IGFBP-3 gene was constructed by annealing and ligating synthetic oligonucleotides, as described in Example 4. The IGFBP-3 sequence used for construction of the gene encoding the DsbA::3C::IGFBP-3 fusion protein was a PCR amplified DNA fragment made using primers BP3RZ and NBP3F and template pYZ42580. Cloning of the two DNA sources used to make chromosomal transfer DNA carrying the gene encoding the DsbA::3C::IGFBP-3 fusion protein, pDM46947 and pDM46948, is shown in Figure 22.

The chromosomal transfer DNA was constructed using ~~EcoRI~~/XbaI-fragments from pDM46947 and pDM46948. The chromosomal transfer DNA was transformed into B1384 cells grown in the presence of indole acrylic acid (to induce the expression of INT). Integrants were selected for by growth of transformants on media containing kanamycin. All kanamycin resistant isolates were also ampicillin sensitive.

What is claimed is:

1. A method for producing a heterologous protein of interest, comprising the steps of:

transferring a chromosomal transfer DNA into a bacterial host cell,

wherein said chromosomal transfer DNA comprising at least one copy of a gene encoding the heterologous protein of interest and a selectable marker,

and wherein said host cell comprising a chromosome;

selecting for integration of said chromosomal transfer DNA into said host cell chromosome resulting in a host cell chromosome comprising a gene encoding a heterologous protein of interest operably linked to a promoter functional in the host cell and a selectable marker flanked by duplicate DNA; and

expressing said gene,

wherein said gene is at no time operably linked to a promoter functional in a host cell on a multicopy number plasmid vector during construction of the transfer DNA and

wherein said non-bacterial protein of interest accumulates within said host cell to a level in excess of 0.1% of total cell protein.

2. The method of claim 1 wherein said chromosomal transfer DNA further comprises a promoter functional in said host cell, said promoter being operably linked to said gene encoding the heterologous protein of interest, and

3. The method of claim 1 wherein said host cell chromosome further comprises a host cell promoter and said chromosomal transfer DNA further comprises a DNA sequence homologous to a segment of the bacterial chromosome downstream from said host cell promoter, said DNA sequence linked in-frame to said gene encoding the heterologous protein of interest,

wherein integration of said chromosomal transfer DNA results in the formation of an operable linkage between said DNA sequence and the host cell promoter.

said first plasmid comprising a first gene encoding a heterologous protein of interest and a first promoter functional in a host cell, and wherein said first gene and first promoter are not operably linked,

said second vector comprising a second gene encoding a heterologous protein of interest lacking an operably linked promoter, and a second promoter functional in a host cell,

wherein said chromosomal transfer DNA comprises a selectable marker and lacks an origin of replication operable in said host cell and wherein said first gene is operably linked to said second promoter on the chromosomal transfer DNA and said second gene is operably linked to said first promoter on said chromosomal transfer DNA.

11. A chromosomal transfer DNA , comprising:

a gene encoding a heterologous protein of interest operably linked to a promoter functional in a host cell; and

a selectable marker, said selectable marker flanked by duplicate DNA,

wherein said gene encoding a heterologous protein of interest is at no time operably linked to a promoter functional in a host cell on a multicopy number plasmid vector.

12. A chromosomal transfer DNA, comprising:

two copies of a gene encoding a heterologous protein of interest, each of said copies being operably linked to a promoter functional in a host cell; and

a selectable marker, said selectable marker flanked by said copies of said gene encoding a heterologous protein of interest,

wherein each of said copies of said gene are at no time operably linked to a promoter functional in a host cell on a multicopy number plasmid vector.

5. The method of claim 1 wherein said heterologous protein of interest is a eukaryotic protein.

6. The method of claim 1 wherein said heterologous protein of interest is a mammalian protein.

7. The method of claim 1 wherein each said duplicate DNA comprises said gene encoding a heterologous protein of interest linked to said promoter.

8. The method of claim 1 further comprising selecting for chromosomal amplification of said chromosomal transfer DNA following integration of said chromosomal transfer DNA into the chromosome of said host cell.

9. A method for producing a chromosomal transfer DNA comprising:

ligating a restriction fragment from each of a first plasmid vector and a second plasmid vector thereby producing said chromosomal transfer DNA, said first vector comprising a gene encoding a heterologous protein of interest lacking an operably linked promoter, said second vector comprising a promoter functional in a host cell, wherein said chromosomal transfer DNA comprises a selectable marker, said gene encoding a heterologous protein of interest operably linked to said promoter and duplicate DNA flanking said gene and lacks an origin of replication operable in said host cell.

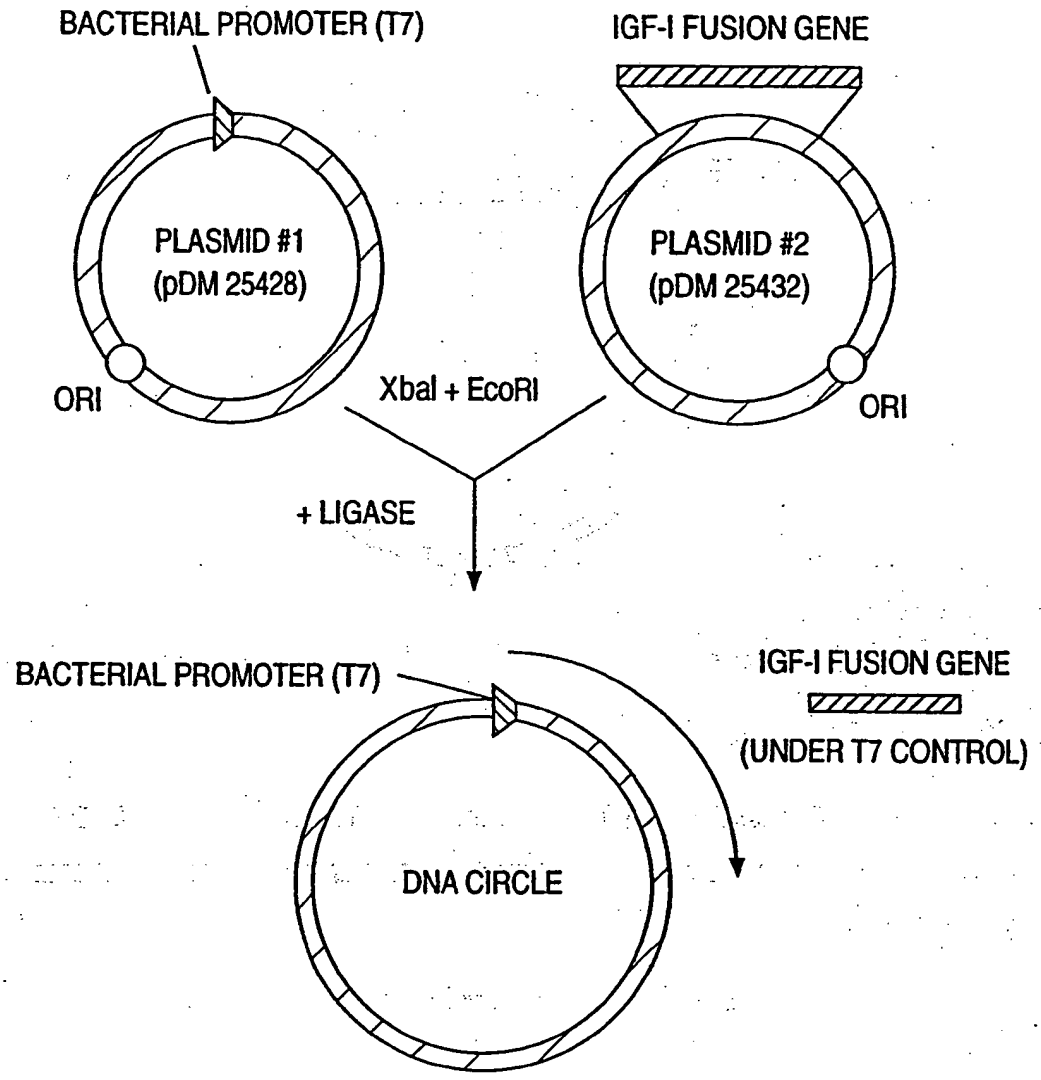


FIG. 1

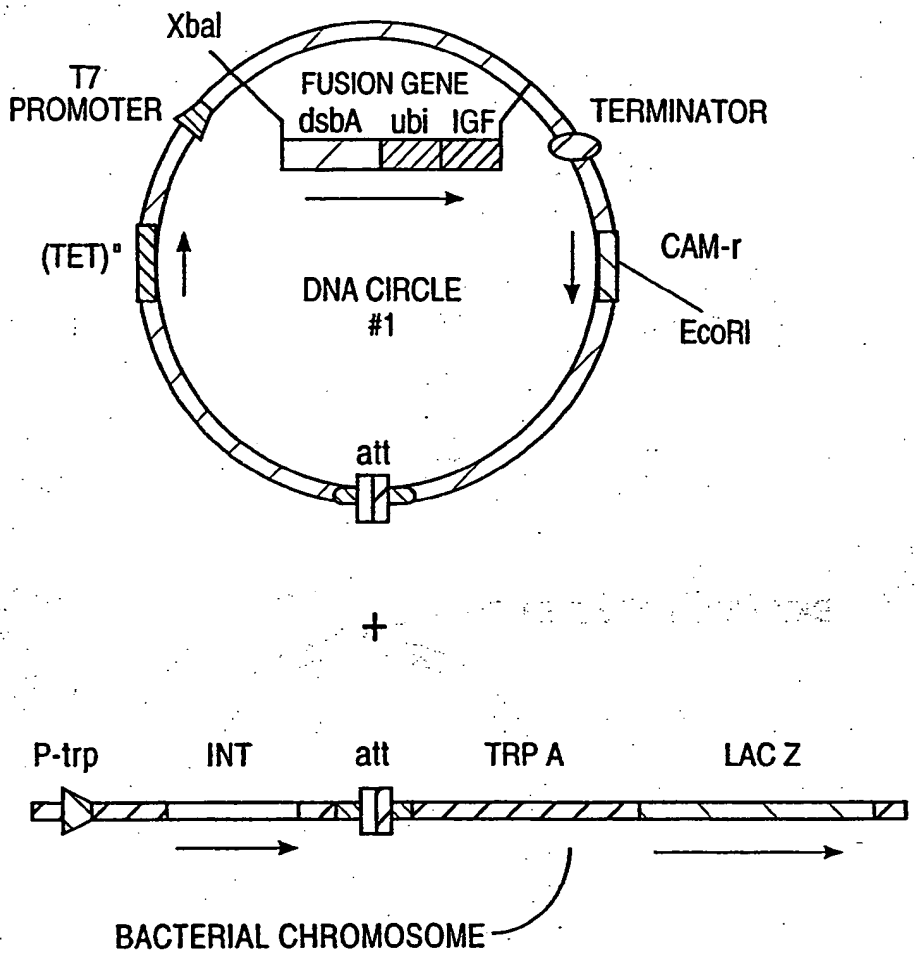

XbaI
T7
PROMOTER
FUSION GENE
dsbA ubi IGF
TERMINATOR
(TET)ᴿ
DNA CIRCLE
#1
CAM-r
EcoRI
att
+
P-trp
INT
att
TRP A
LAC Z
BACTERIAL CHROMOSOME

FIG. 2

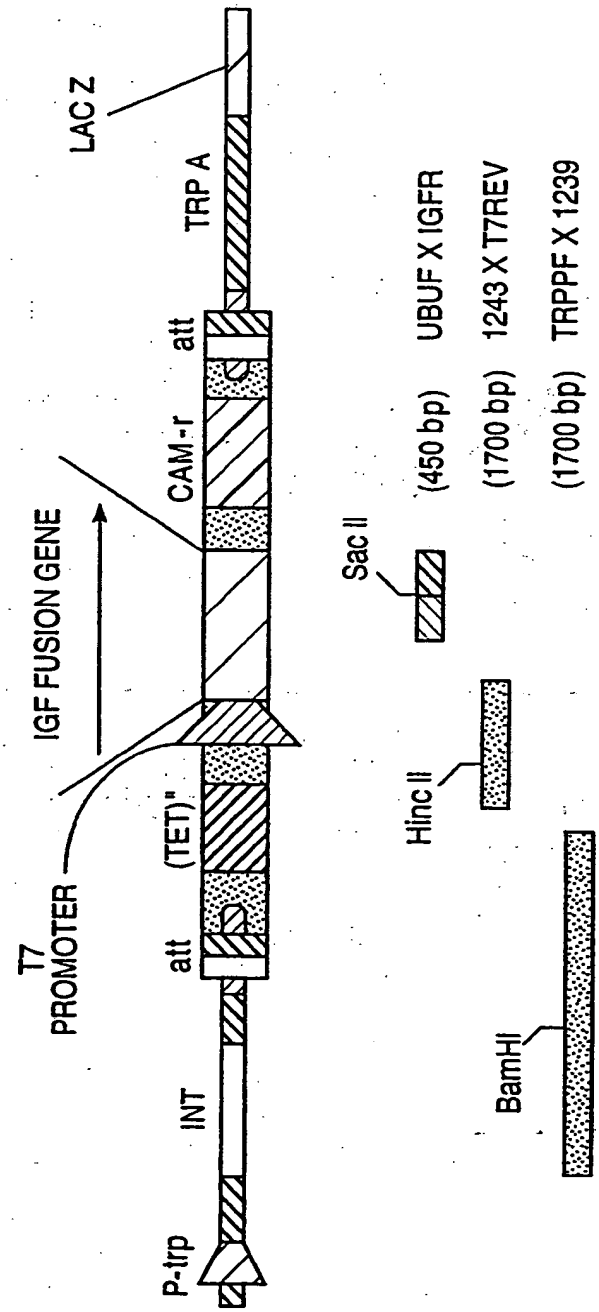


FIG. 3

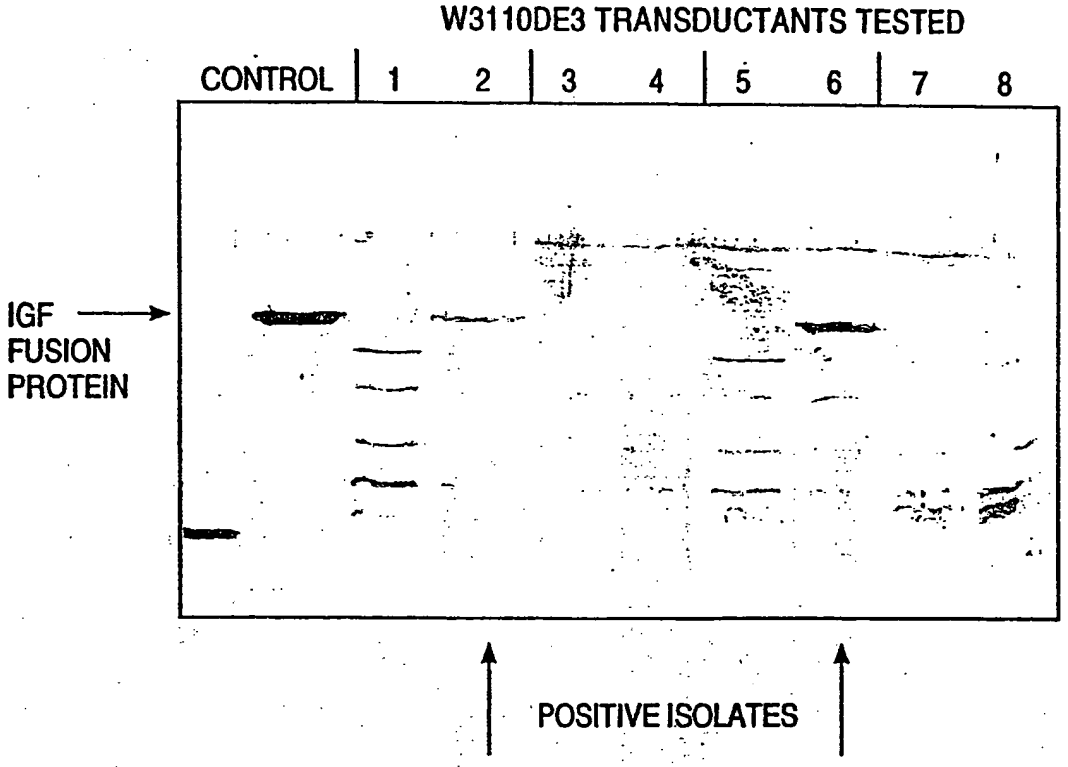


FIG. 4

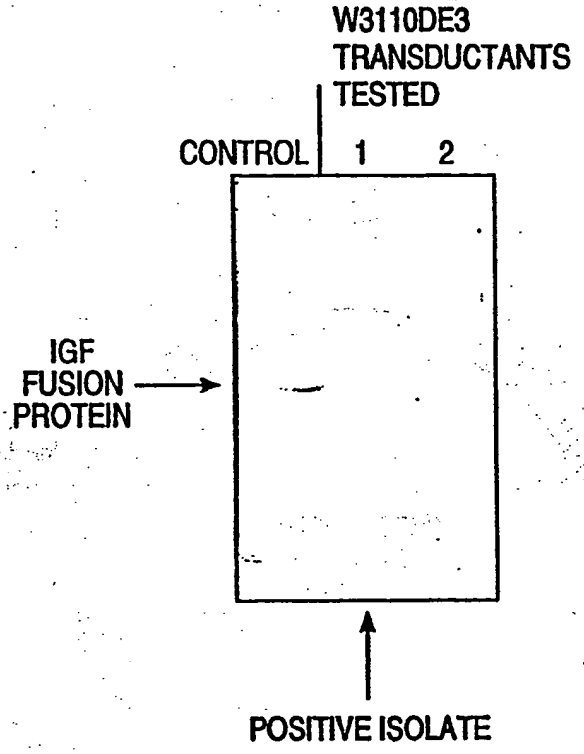
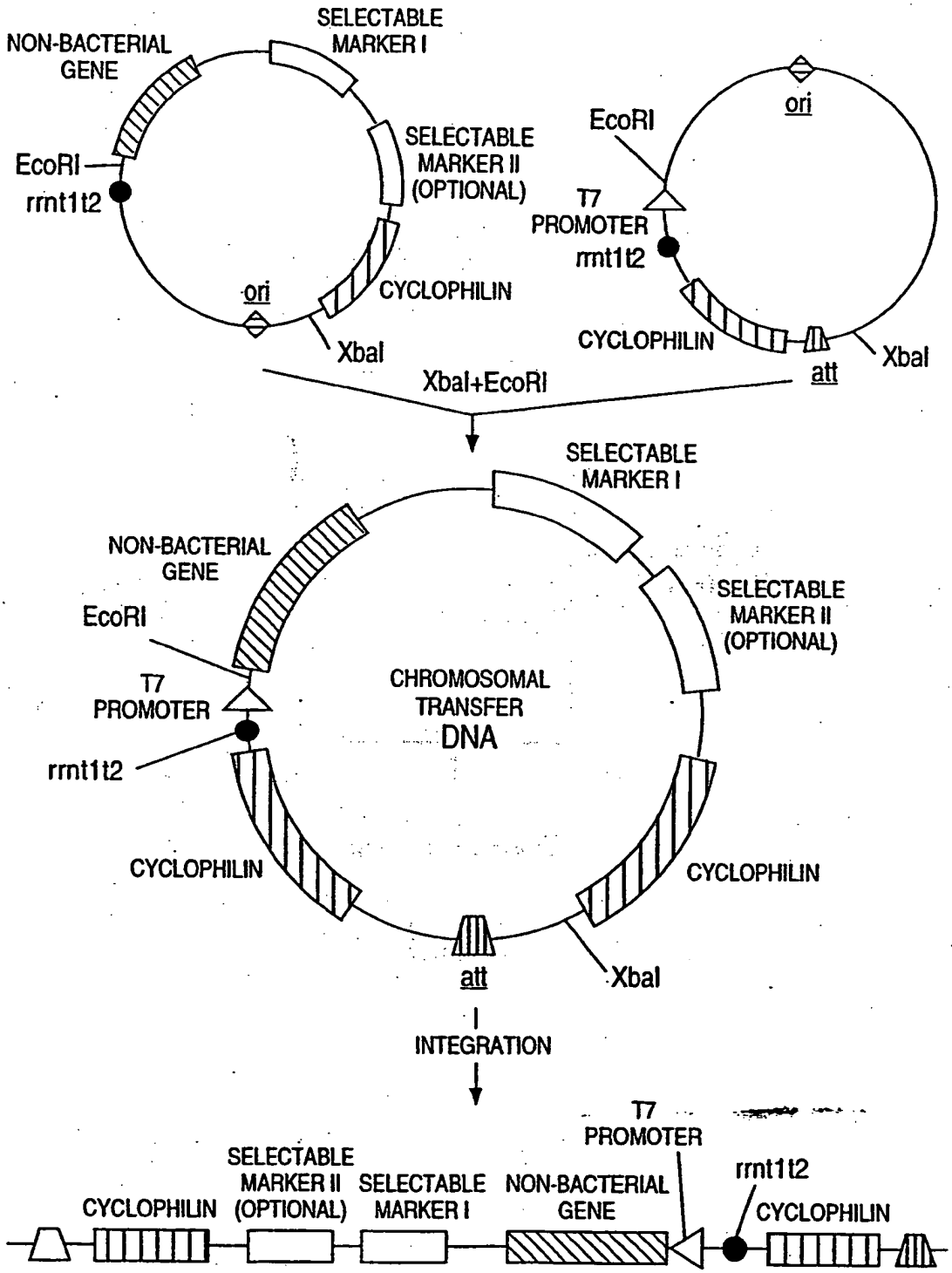


FIG. 5


SELECTABLE MARKER I
NON-BACTERIAL GENE
EcoRI
rrnt1t2
SELECTABLE MARKER II (OPTIONAL)
ori
CYCLOPHILIN
XbaI
EcoRI
ori
T7 PROMOTER
rrnt1t2
CYCLOPHILIN
XbaI
att
XbaI+EcoRI
SELECTABLE MARKER I
NON-BACTERIAL GENE
EcoRI
T7 PROMOTER
rrnt1t2
CHROMOSOMAL TRANSFER DNA
SELECTABLE MARKER II (OPTIONAL)
CYCLOPHILIN
CYCLOPHILIN
att
XbaI
INTEGRATION
T7 PROMOTER
rrnt1t2
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SELECTABLE MARKER II (OPTIONAL)
SELECTABLE MARKER I
NON-BACTERIAL GENE
CYCLOPHILIN

FIG. 6

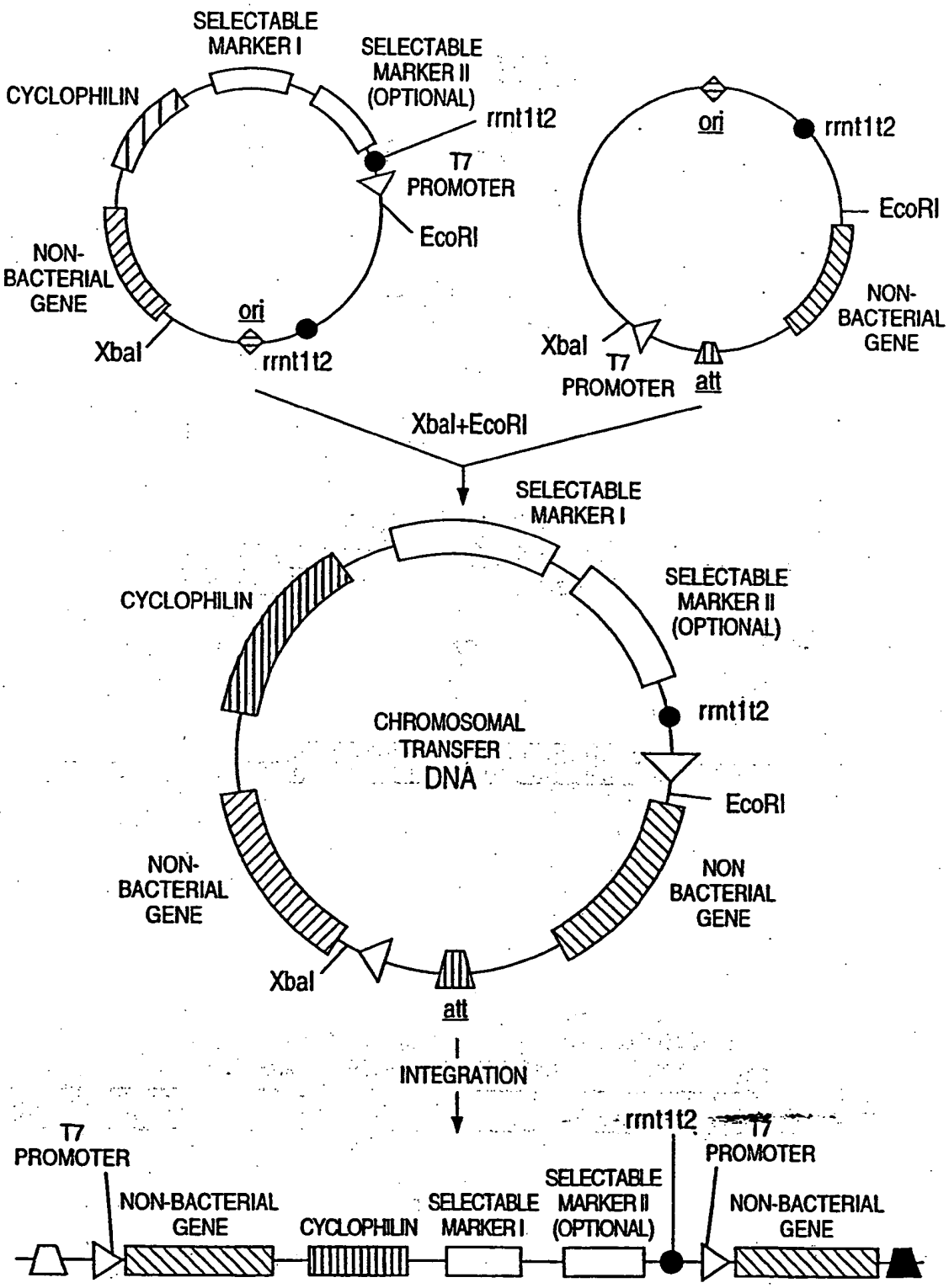
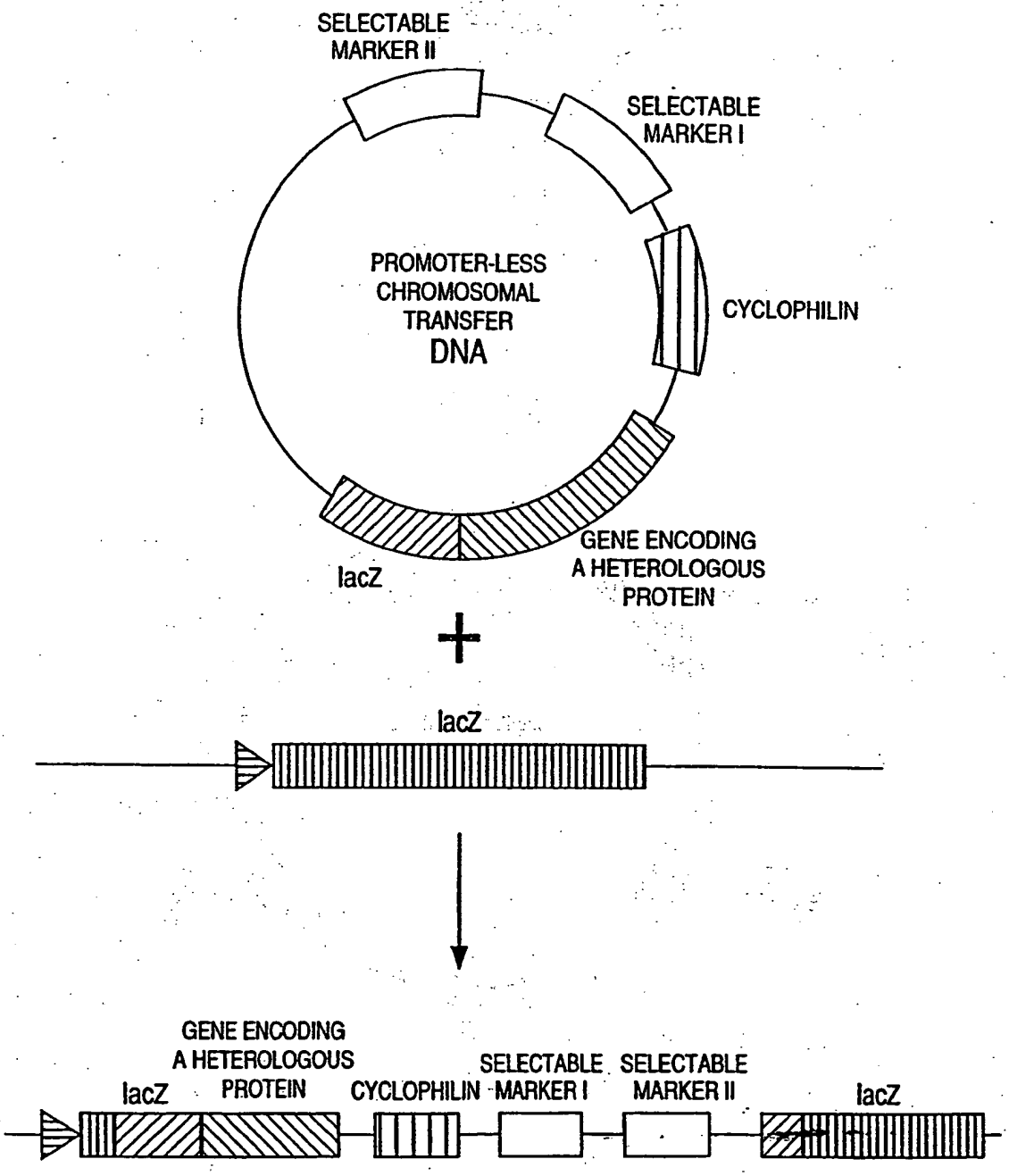


FIG. 7

SUBSTITUTE SHEET (RULE 26)


SELECTABLE MARKER II
SELECTABLE MARKER I
PROMOTER-LESS CHROMOSOMAL TRANSFER DNA
CYCLOPHILIN
lacZ
GENE ENCODING A HETEROLOGOUS PROTEIN
lacZ
GENE ENCODING A HETEROLOGOUS PROTEIN
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CYCLOPHILIN
SELECTABLE MARKER I
SELECTABLE MARKER II
lacZ

FIG. 8

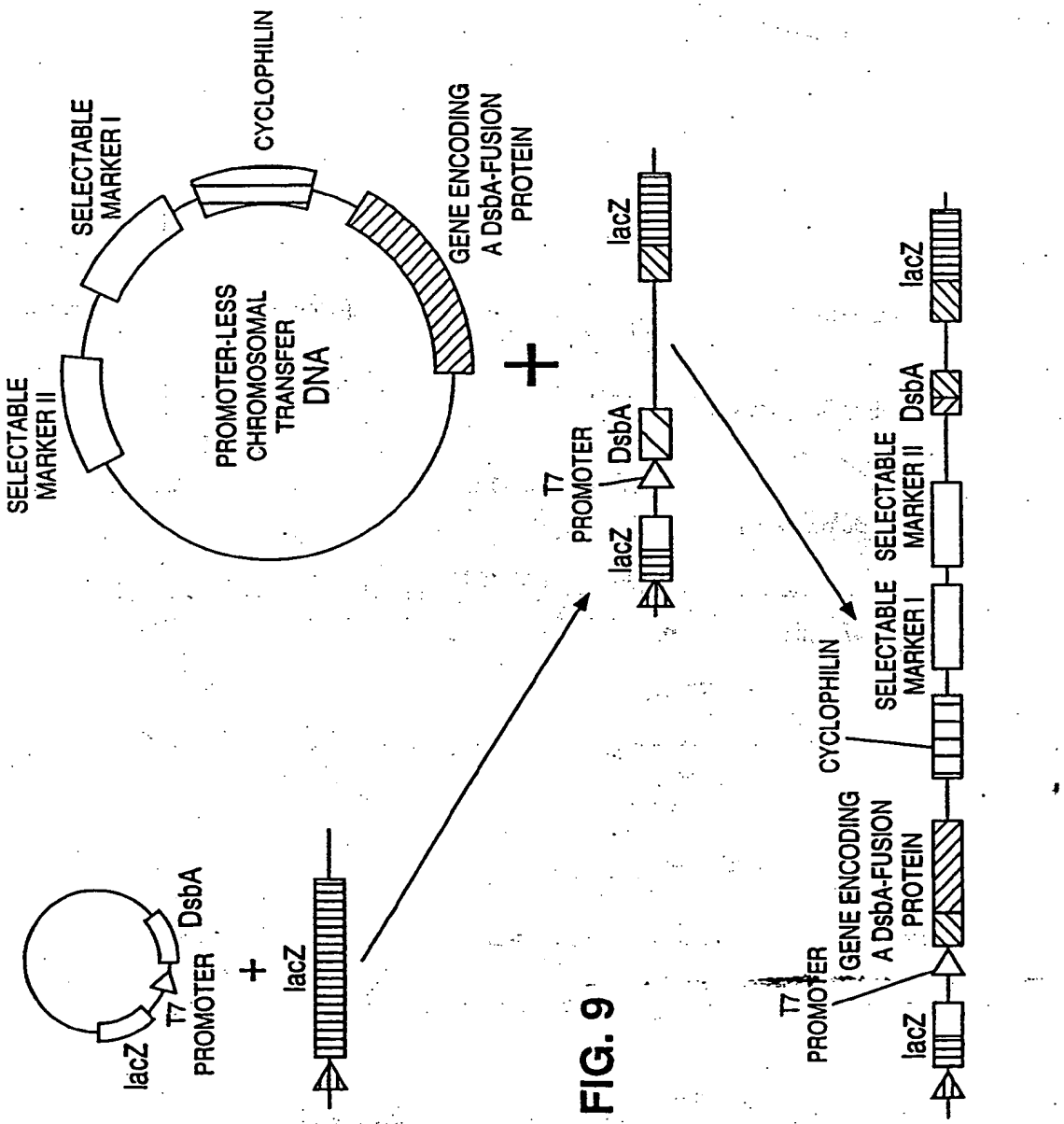

FIG. 9

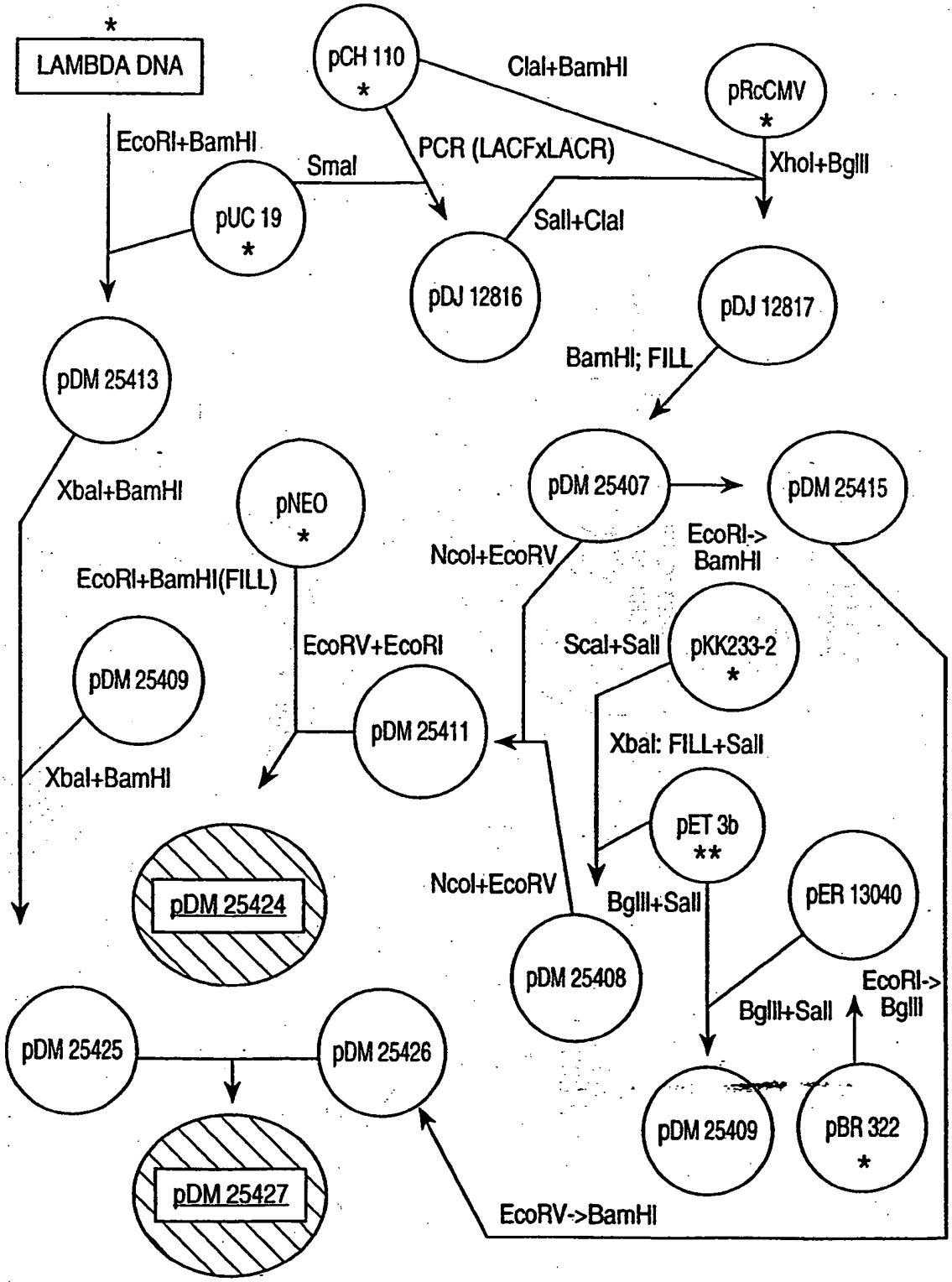

FIG. 10

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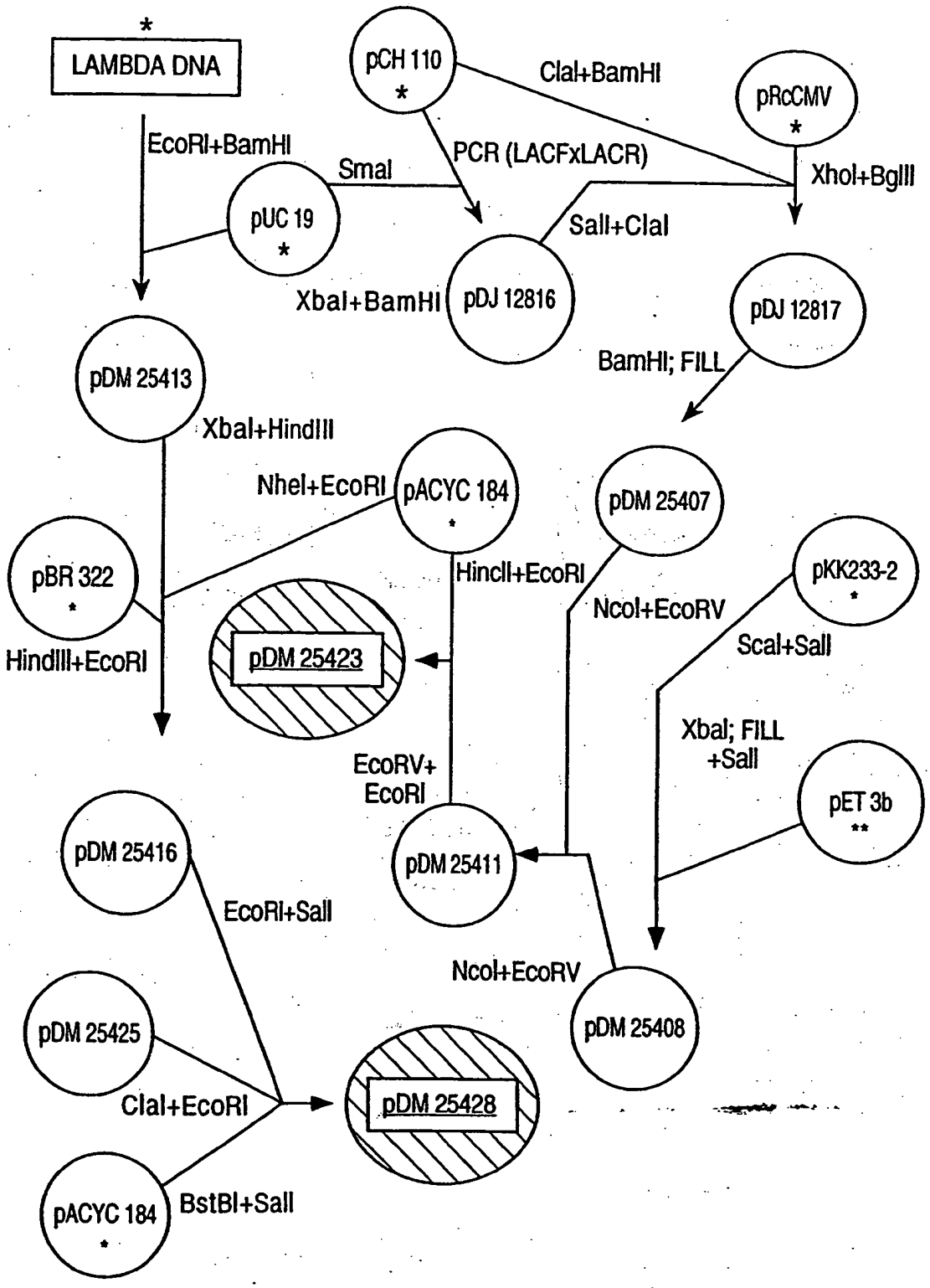
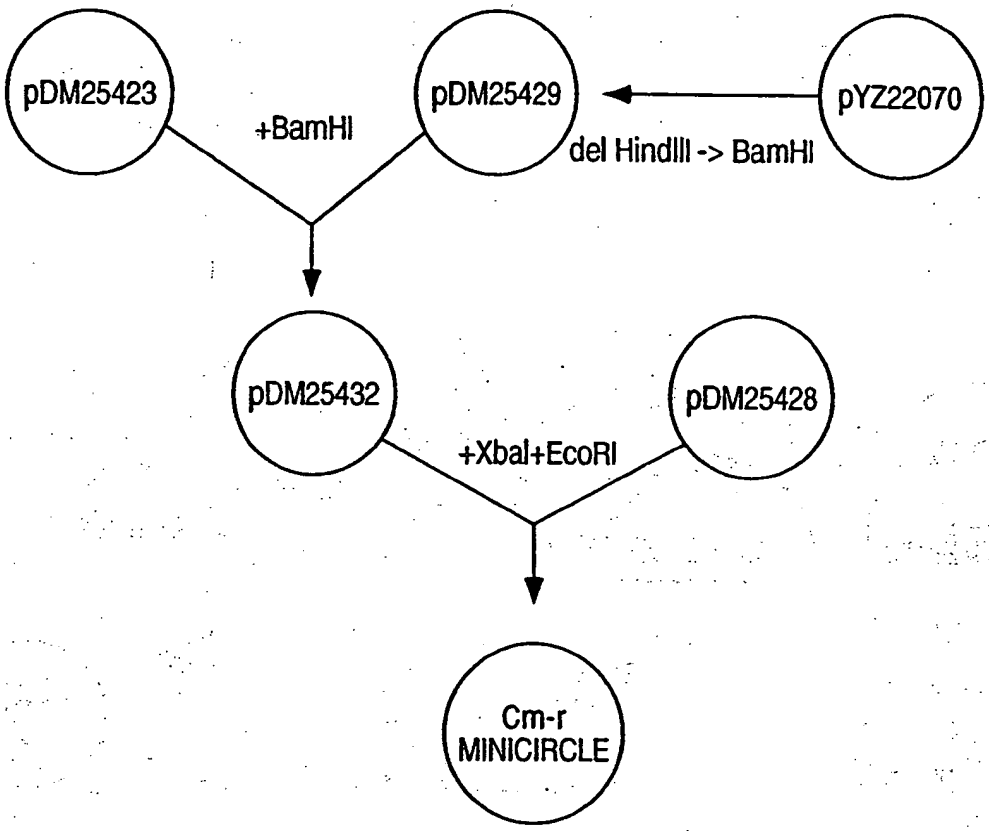


FIG. 11
RECTIFIED SHEET (RULE 91)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+BamHI
pDM25429
del HindIII -> BamHI
pYZ22070
pDM25432
+XbaI+EcoRI
pDM25428
Cm-r
MINICIRCLE

FIG. 12

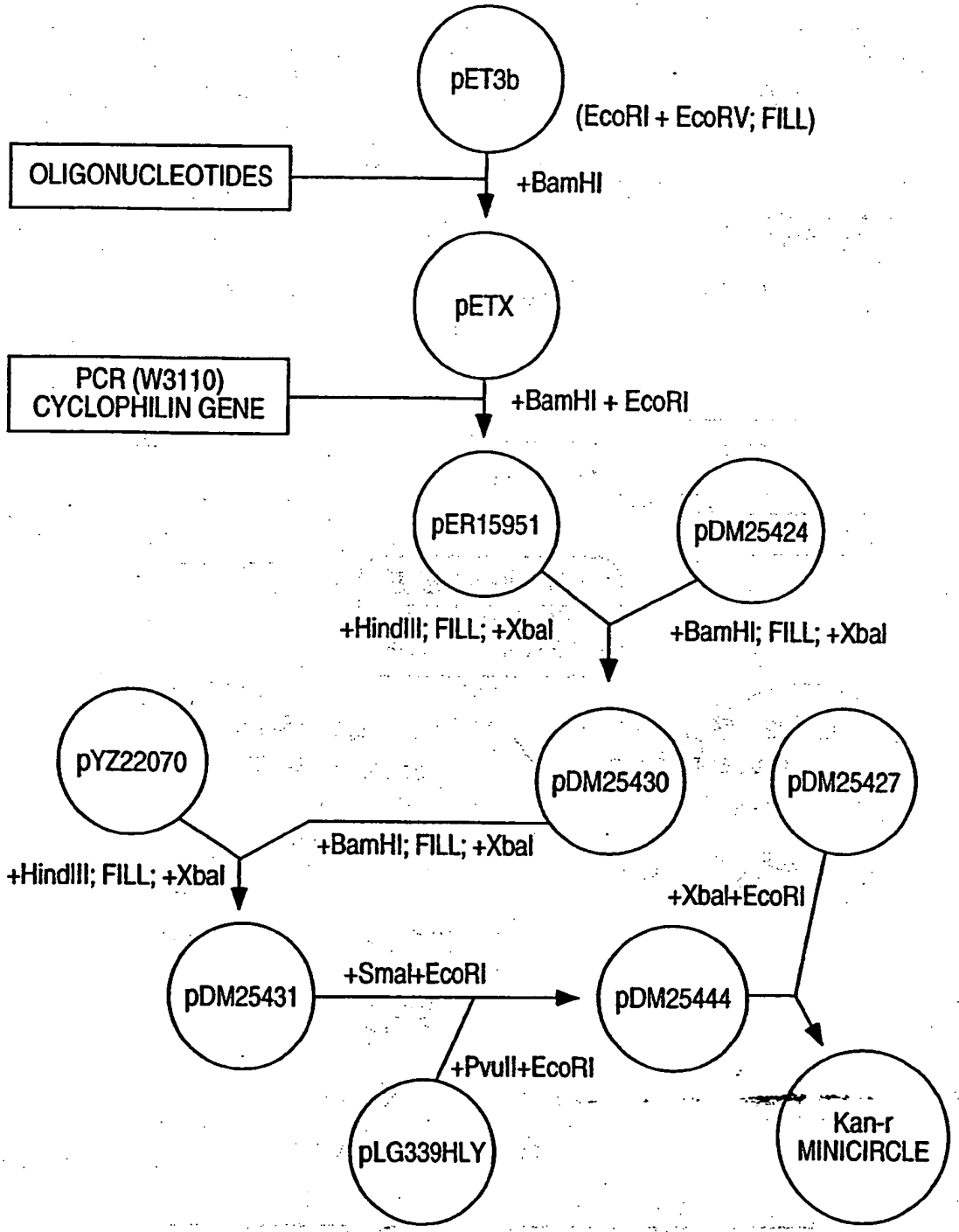


FIG. 13

RECTIFIED SHEET (RULE 91)

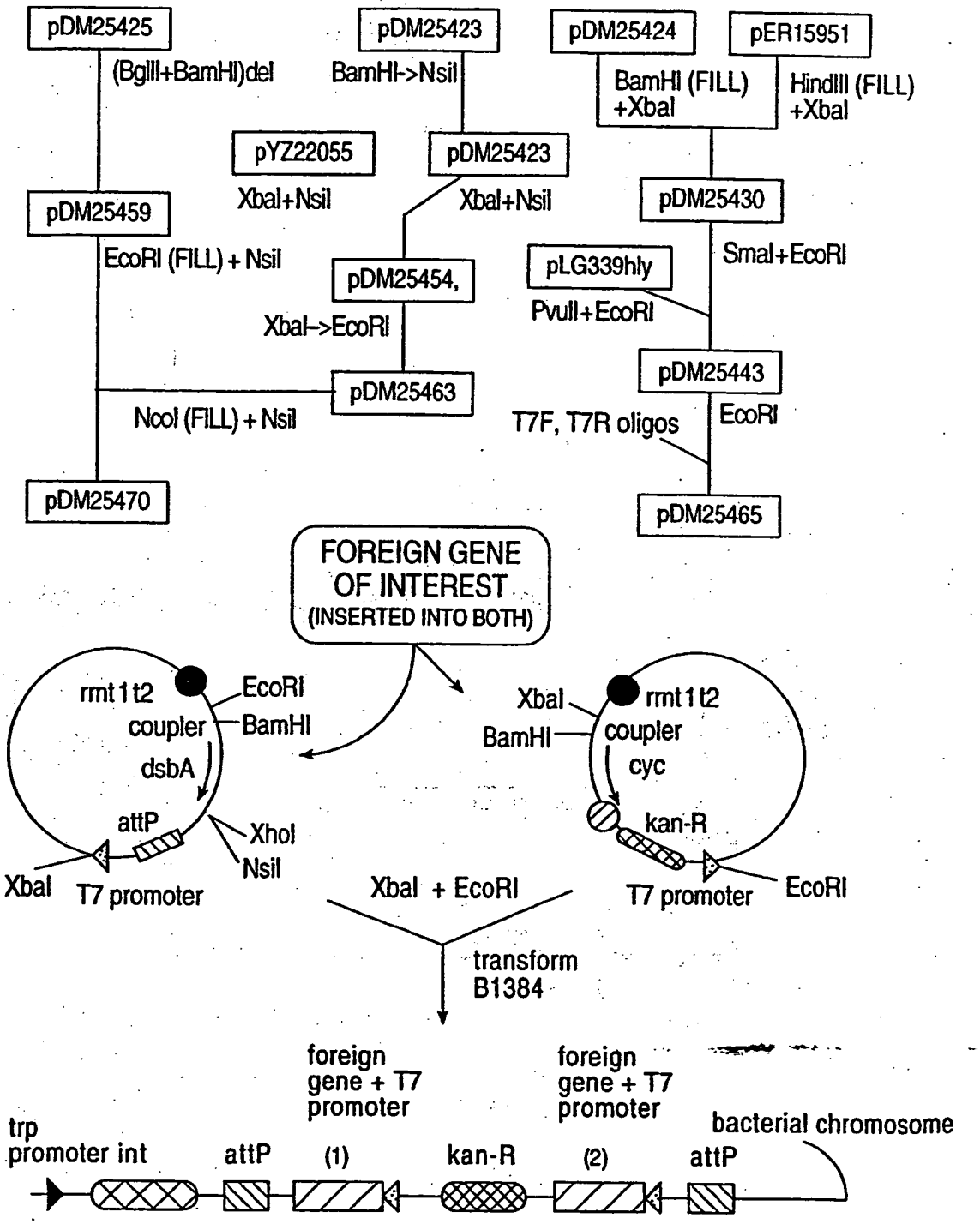


FIG. 14

RECTIFIED SHEET (RULE 91)

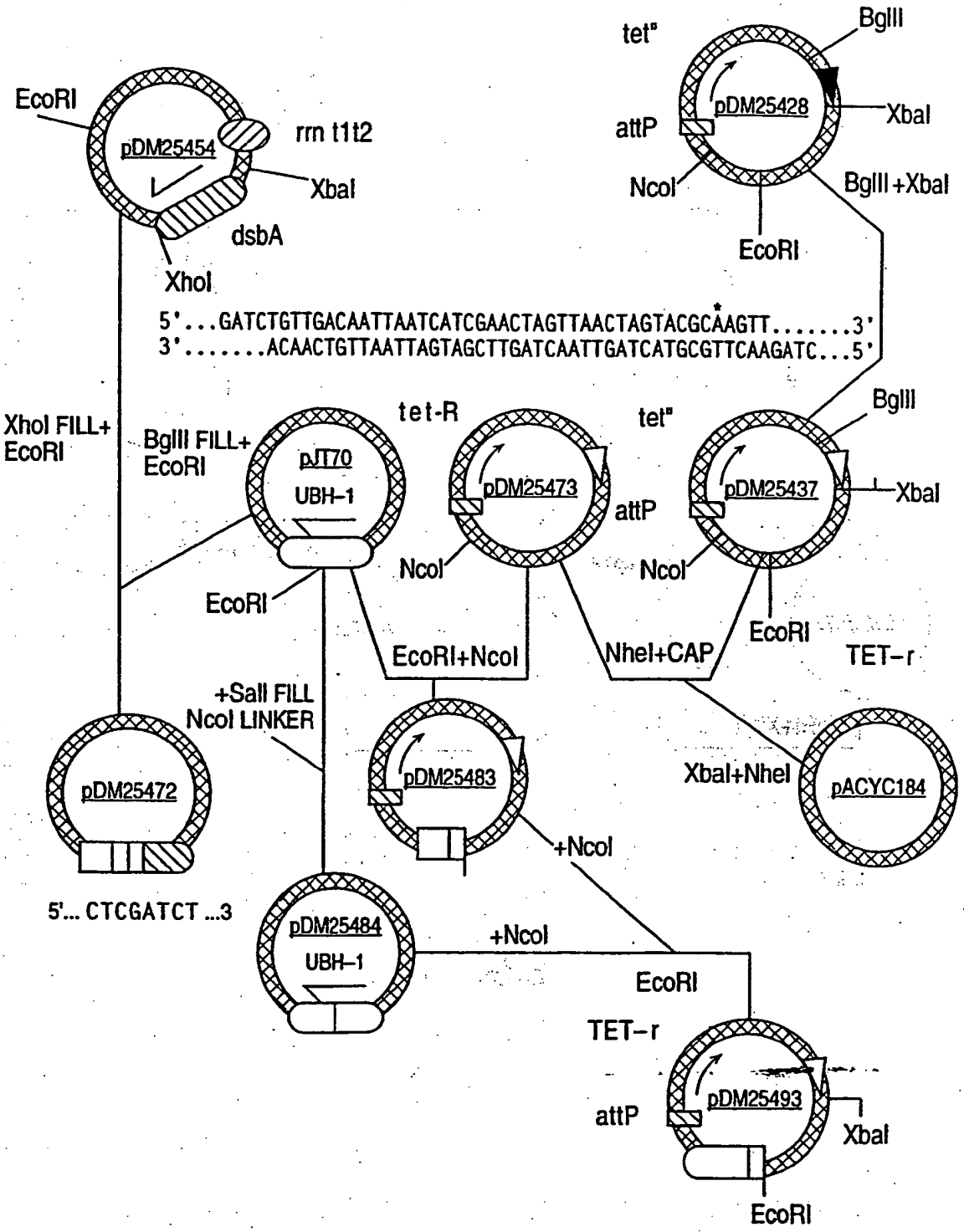
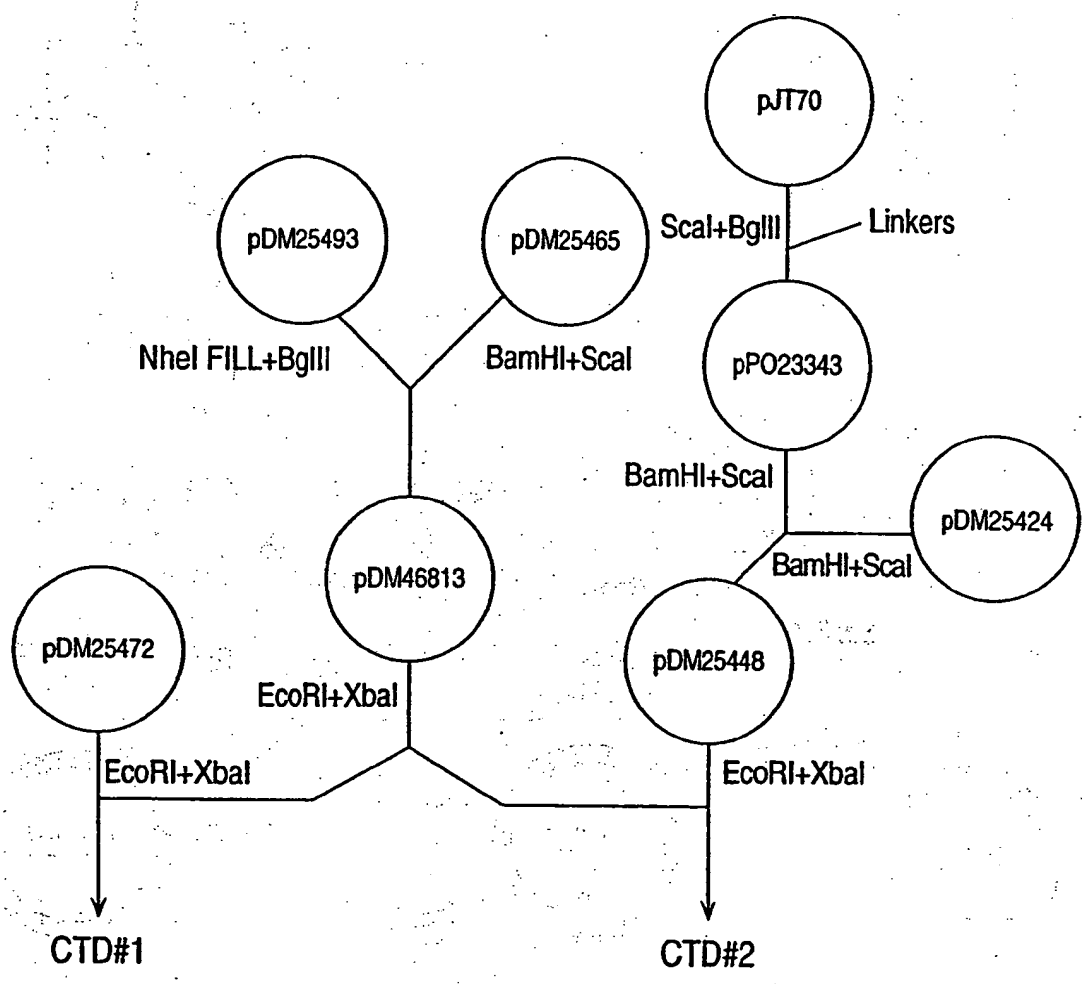


FIG. 15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pDM25465
ScaI+BglII
Linkers
NheI FILL+BglII
BamHI+ScaI
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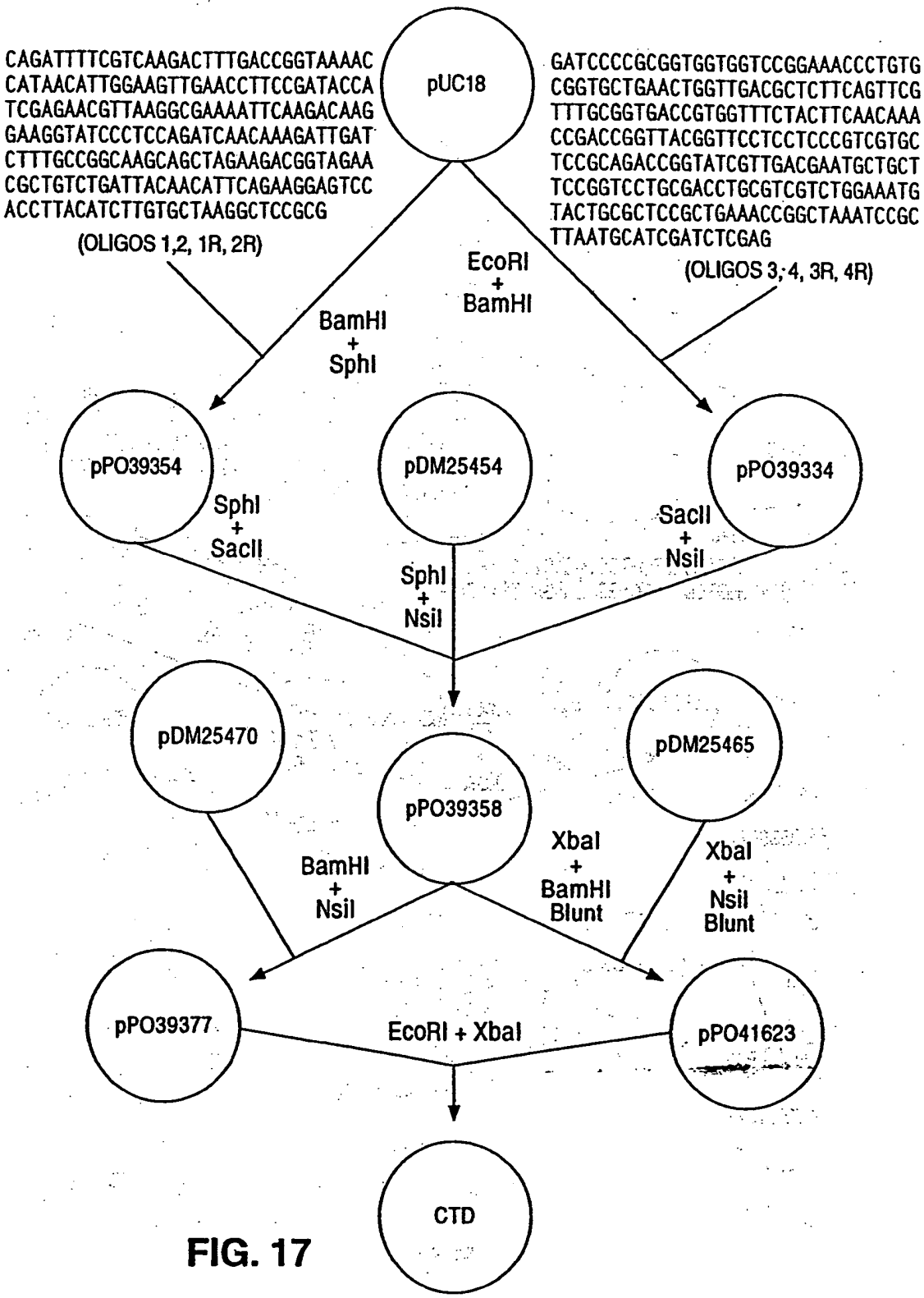
pDM25424
pDM46813
BamHI+ScaI
pDM25472
pDM25448
EcoRI+XbaI
EcoRI+XbaI
EcoRI+XbaI
CTD#1
CTD#2

FIG. 16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CGCTGTCTGATTACAACATTCAGAAGGAGTCC
ACCTTACATCTTGTGCTAAGGCTCCGCG
(OLIGOS 1,2, 1R, 2R)
pUC18
GATCCCCGCGGTGGTGGTCCGGAAACCCTGTG
CGGTGCTGAACTGGTTGACGCTCTTCAGTTCG
TTTGCGGTGACCGTGGTTTCTACTTCAACAAA
CCGACCGGTTACGGTTCCTCCTCCCGTCGTGC
TCCGCAGACCGGTATCGTTGACGAATGCTGCT
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TACTGCGCTCCGCTGAAACCGGCTAAATCCGC
TTAATGCATCGATCTCGAG
(OLIGOS 3, 4, 3R, 4R)
BamHI + SphI
EcoRI + BamHI
pPO39354
pDM25454
pPO39334
SphI + SacII
SacII + NsiI
SphI + NsiI
pDM25470
pPO39358
pDM25465
BamHI + NsiI
XbaI + BamHI Blunt
XbaI + NsiI Blunt
pPO39377
EcoRI + XbaI
pPO41623
CTD

FIG. 17

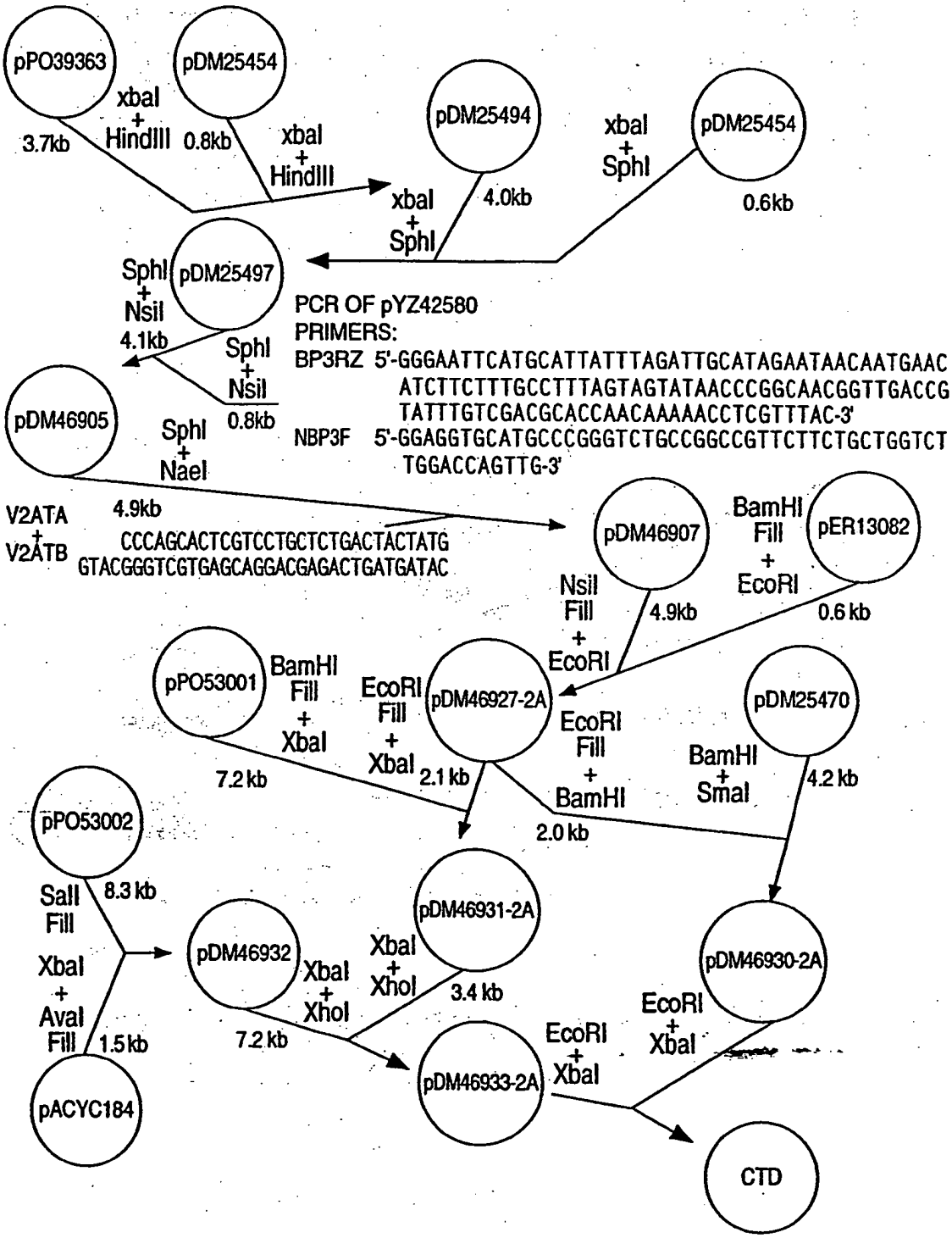

pPO39363
pDM25454
XbaI + HindIII
3.7kb
0.8kb
XbaI + HindIII
pDM25494
XbaI + SphI
4.0kb
XbaI + SphI
pDM25454
0.6kb
SphI + NsiI
pDM25497
4.1kb
SphI + NsiI
0.8kb
PCR OF pYZ42580
PRIMERS:
BP3RZ 5'-GGGAATTCATGCATTATTTAGATTGCATAGAATAACAATGAAC
ATCTTCTTTGCCTTTAGTAGTATAACCCGGCAACGGTTGACCG
TATTTGTCGACGCACCAACAAAAACCTCGTTTAC-3'
NBP3F 5'-GGAGGTGCATGCCCGGGTCTGCCGGCCGTTCTTCTGCTGGTCT
TGGACCAGTTG-3'
pDM46905
SphI + NaeI
4.9kb
V2ATA + V2ATB
CCCAGCACTCGTCCTGCTCTGACTACTATG
GTACGGGTCGTGAGCAGGACGAGACTGATGATAC
pDM46907
NsiI FilI + EcoRI
4.9kb
BamHI FilI + EcoRI
pER13082
0.6 kb
pPO53001
BamHI FilI + XbaI
7.2 kb
EcoRI FilI + XbaI
pDM46927-2A
2.1 kb
EcoRI FilI + BamHI
2.0 kb
pDM25470
BamHI + SmaI
4.2 kb
pPO53002
SalI FilI
8.3 kb
XbaI + AvaI FilI
1.5 kb
pACYC184
pDM46932
XbaI + XhoI
7.2 kb
pDM46931-2A
XbaI + XhoI
3.4 kb
pDM46933-2A
EcoRI + XbaI
pDM46930-2A
EcoRI + XbaI
CTD

FIG. 18

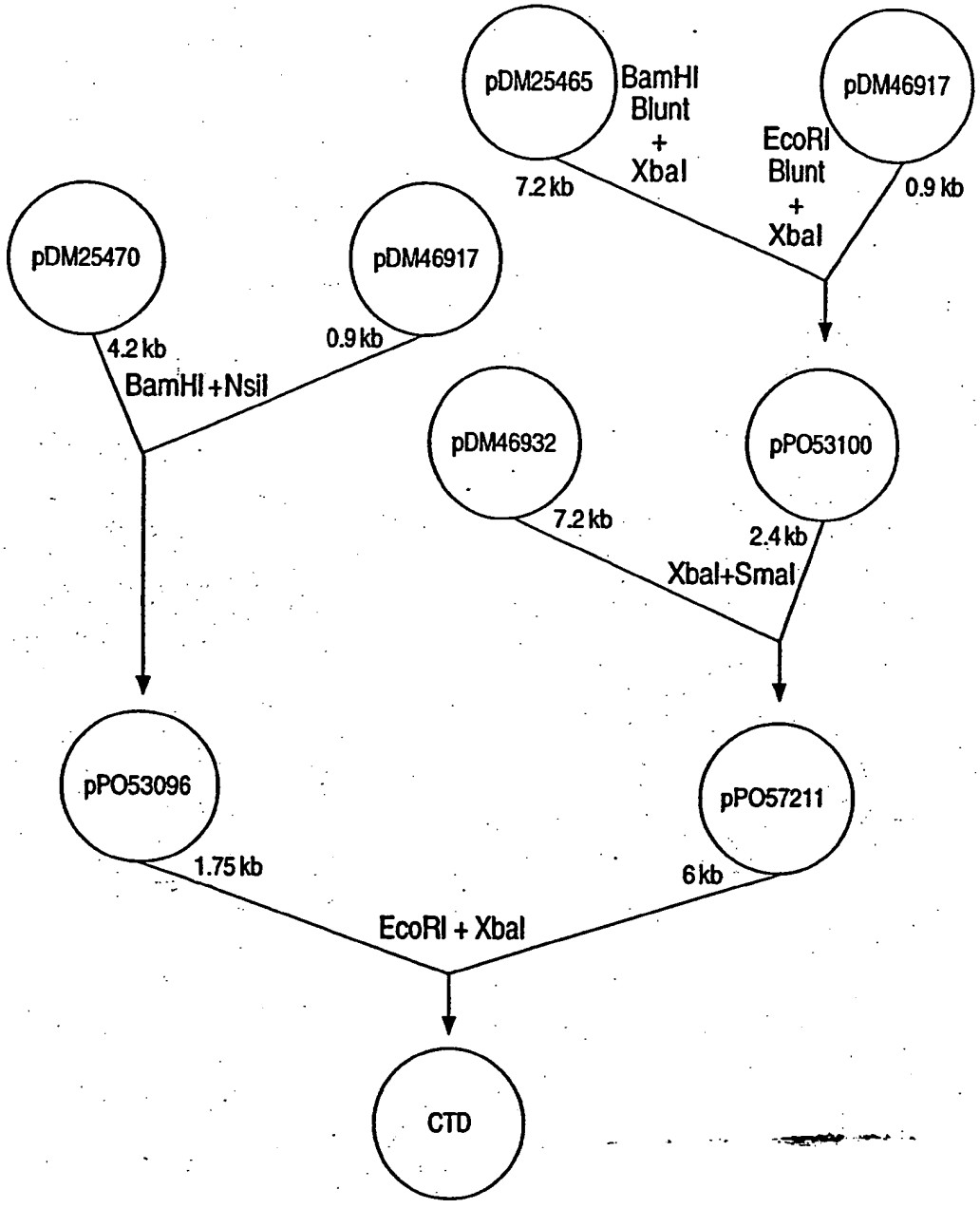

pDM25465
BamHI
Blunt
+
XbaI
7.2 kb
pDM46917
EcoRI
Blunt
+
XbaI
0.9 kb
pDM25470
4.2 kb
BamHI + NsiI
pDM46917
0.9 kb
pDM46932
7.2 kb
pPO53100
2.4 kb
XbaI+SmaI
pPO53096
1.75 kb
pPO57211
6 kb
EcoRI + XbaI
CTD

FIG. 19

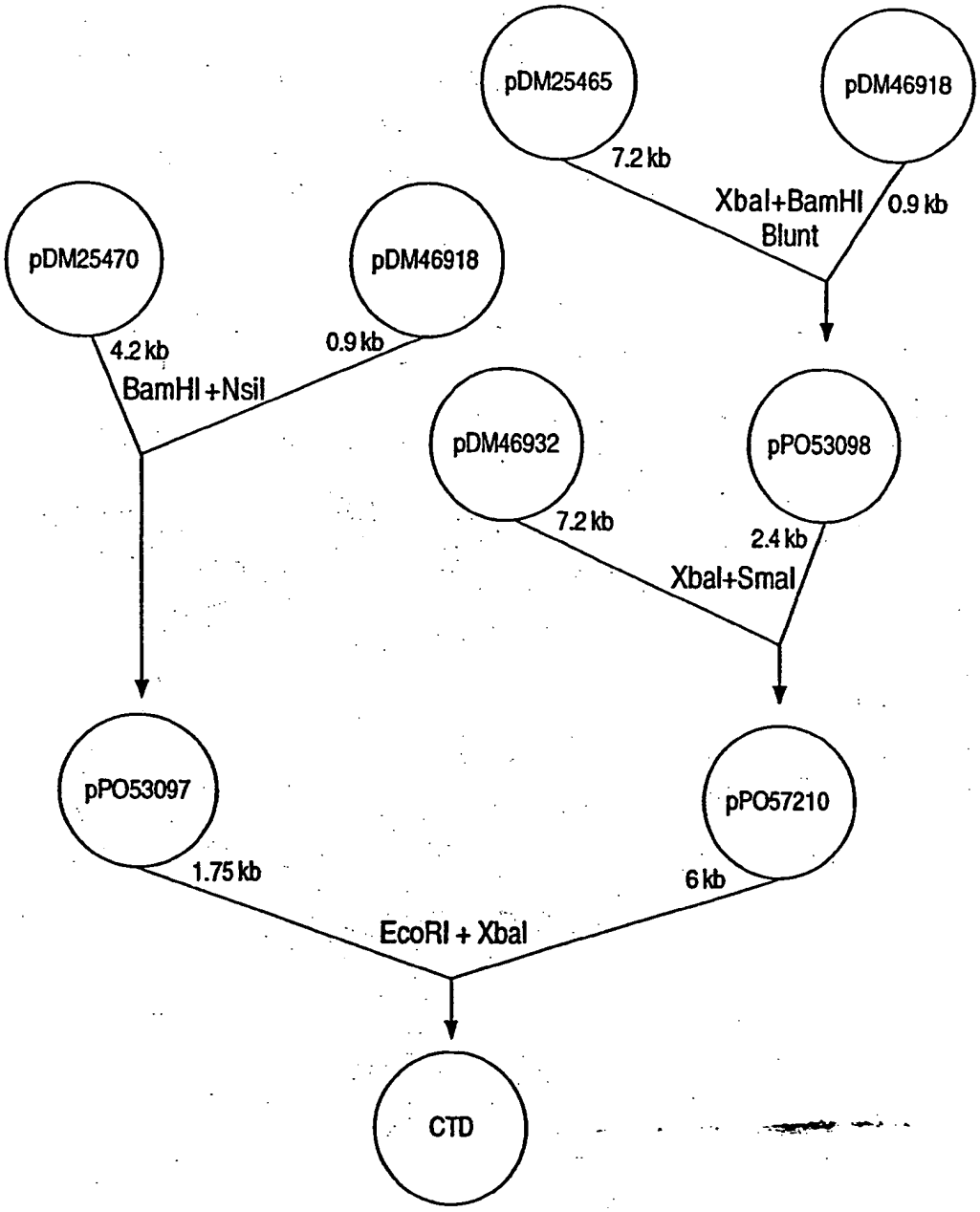

pDM25465
pDM46918
7.2 kb
XbaI+BamHI
Blunt
0.9 kb
pDM25470
pDM46918
4.2 kb
BamHI+NsiI
0.9 kb
pDM46932
pPO53098
7.2 kb
2.4 kb
XbaI+SmaI
pPO53097
pPO57210
1.75 kb
6 kb
EcoRI + XbaI
CTD

FIG. 20

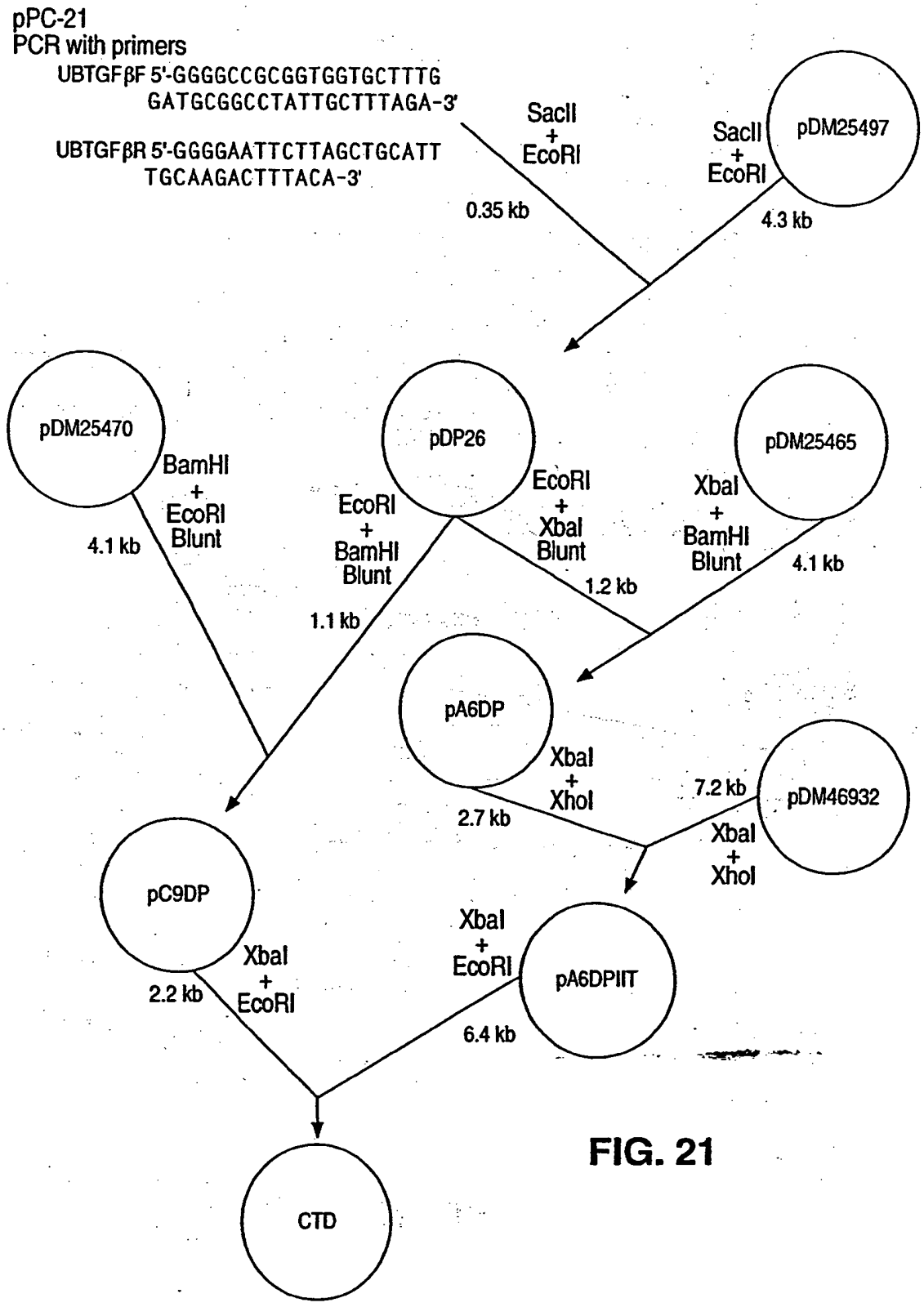


FIG. 21

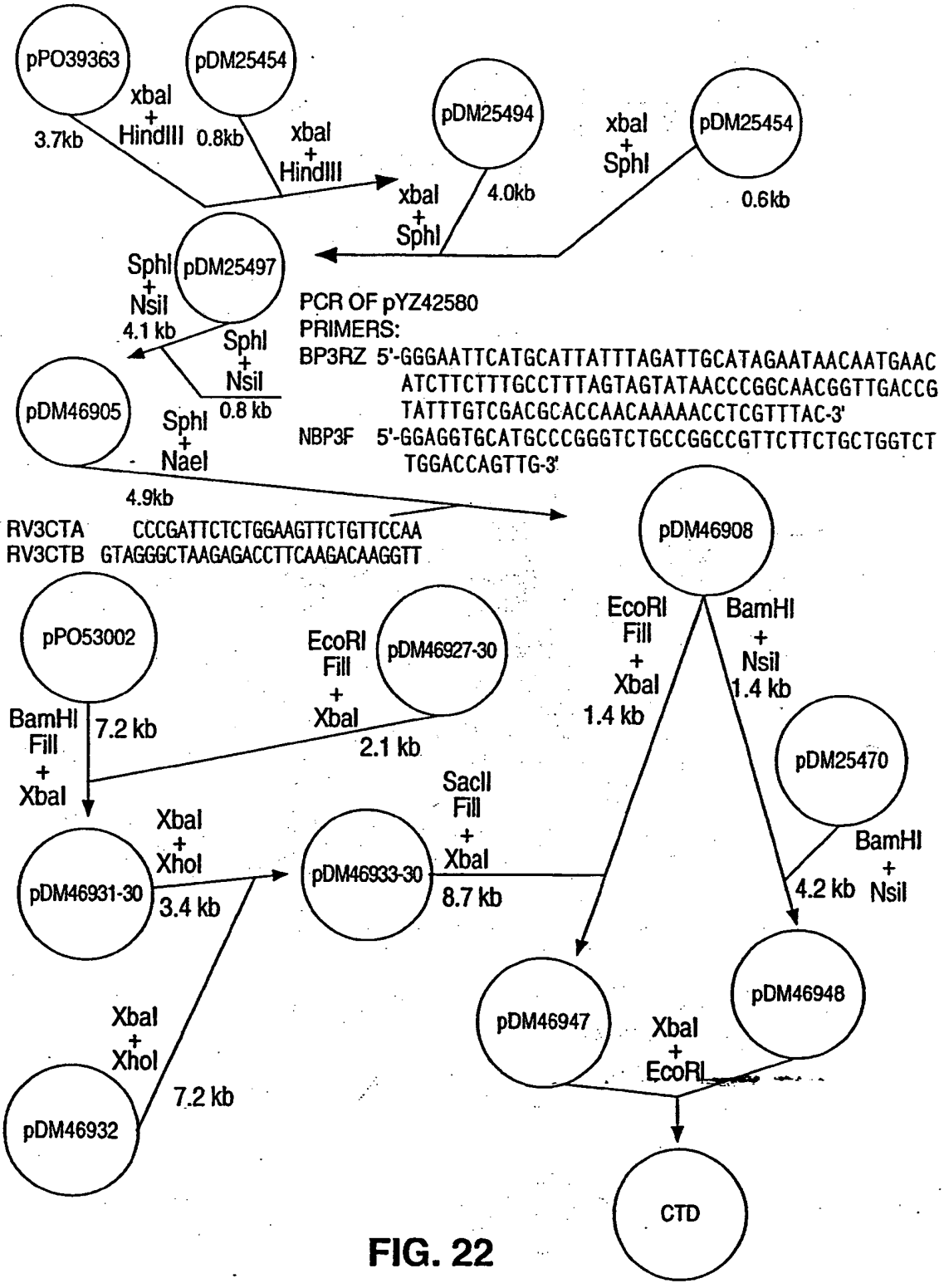

pPO39363
pDM25454
xbal + HindIII
3.7kb
0.8kb
xbal + HindIII
pDM25494
4.0kb
xbal + SphI
pDM25454
0.6kb
xbal + SphI
SphI + NsiI
pDM25497
4.1 kb
SphI + NsiI
0.8 kb
PCR OF pYZ42580
PRIMERS:
BP3RZ 5'-GGGAATTCATGCATTATTTAGATTGCATAGAATAACAATGAAC
ATCTTCTTTGCCTTTAGTAGTATAACCCGGCAACGGTTGACCG
TATTTGTCGACGCACCAACAAAAACCTCGTTTAC-3'
NBP3F 5'-GGAGGTGCATGCCCGGGTCTGCCGGCCGTTCTTCTGCTGGTCT
TGGACCAGTTG-3'
pDM46905
SphI + NaeI
4.9kb
RV3CTA CCCGATTCTCTGGAAGTTCTGTTCCAA
RV3CTB GTAGGGCTAAGAGACCTTCAAGACAAGGTT
pDM46908
pPO53002
EcoRI FilI + XbaI
pDM46927-30
2.1 kb
BamHI FilI + XbaI
7.2 kb
EcoRI FilI + XbaI
1.4 kb
BamHI + NsiI
1.4 kb
pDM25470
BamHI + NsiI
4.2 kb
SacII FilI + XbaI
8.7 kb
XbaI + XhoI
pDM46931-30
3.4 kb
pDM46933-30
pDM46947
pDM46948
XbaI + EcoRI
XbaI + XhoI
7.2 kb
pDM46932
CTD

FIG. 22

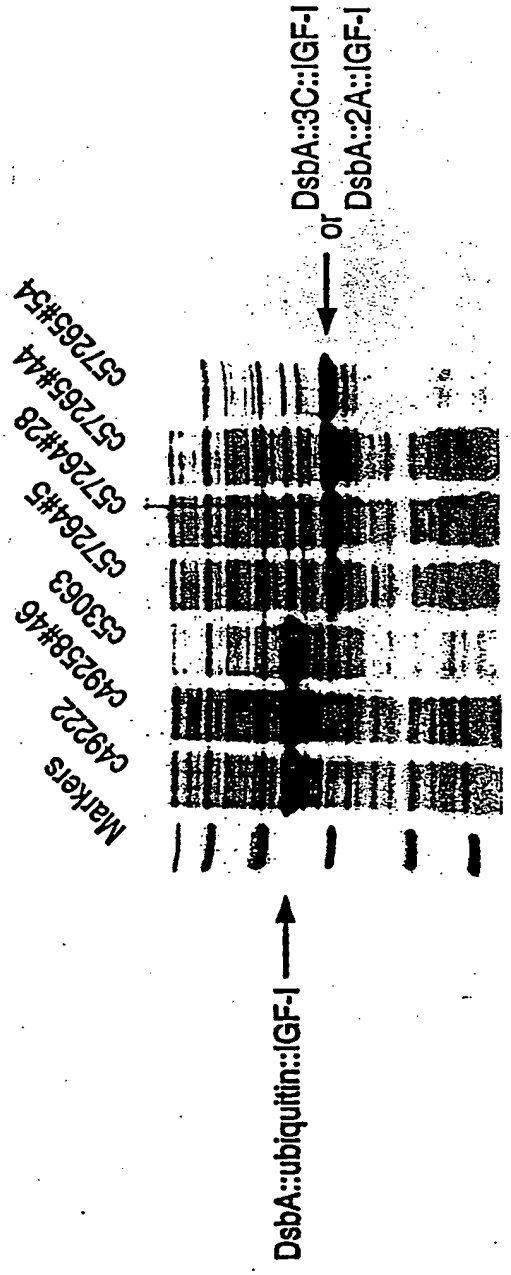


FIG. 23

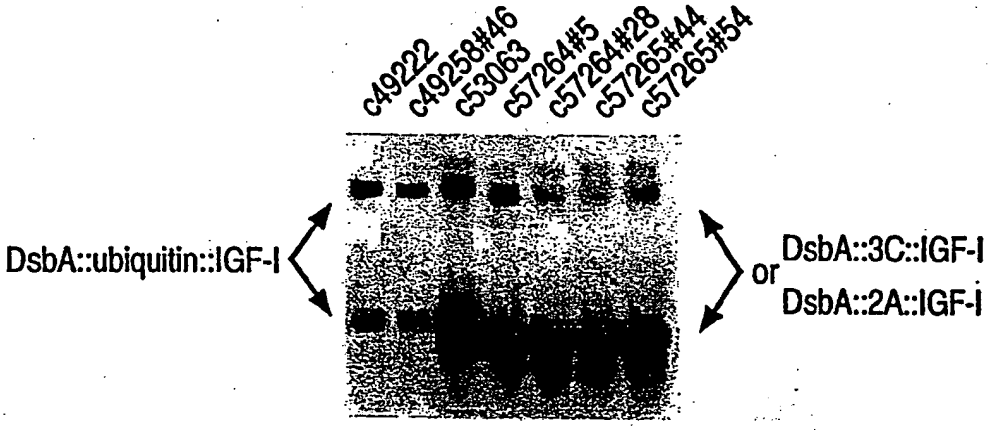

c49222
c49258#46
c53063
c57264#5
c57264#28
c57265#44
c57265#54
DsbA::ubiquitin::IGF-I
or
DsbA::3C::IGF-I
DsbA::2A::IGF-I

FIG. 24

FIG. 25A

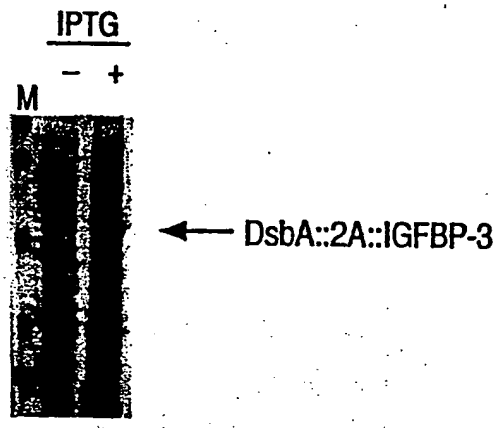
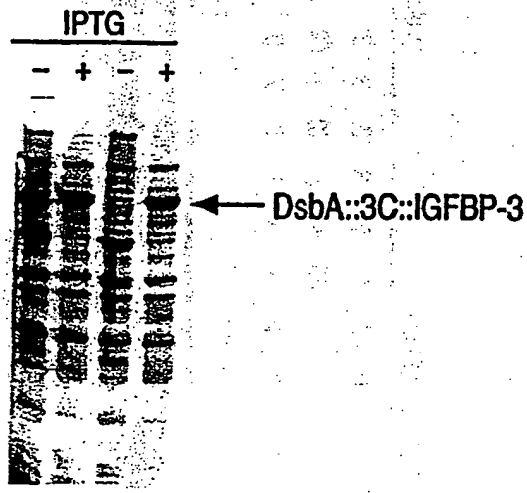


FIG. 25B



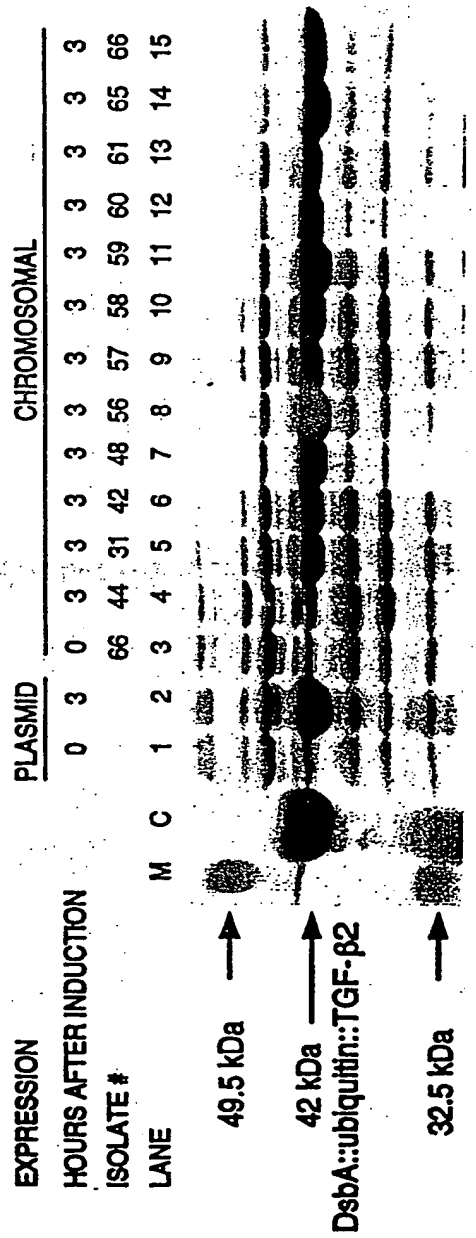

EXPRESSION
HOURS AFTER INDUCTION
ISOLATE #
LANE
M C 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
PLASMID
0 3 0 3 3 3 3 3 3 3 3 3 3 3 3 3
66 44 31 42 48 56 57 58 59 60 61 65 66
CHROMOSOMAL
49.5 kDa
42 kDa
DsbA::ubiquitin::TGF-β2
32.5 kDa

FIG. 26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09006

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12N 15/12, 15/63, 15/64, 15/70, 15/74, 15/90
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 71.1, 172.1, 172.3, 252.3, 252.33, 320.1; 536/23.1, 24.1; 935/22, 27, 33, 38, 41, 42, 52, 72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, DERWENT
search terms: chromosom? transfer?, integrat?, express?, protein?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DIEDERICH et al. New Cloning Vectors for Integration into the λ Attachment Site *attB* of the *Escherichia coli* Chromosome. Plasmid. 1992, Vol. 28, pages 14-24, see entire document.	1-12
A, P	US 5,470,727 A (D. MASCARENHAS ET AL.) 28 November 1995 (28.11.95), see entire document.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 JULY 1996	Date of mailing of the international search report 02 AUG 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY J. DEGEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09006

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 71.1, 172.1, 172.3, 252.3, 252.33, 320.1; 536/23.1, 24.1; 935/22, 27, 33, 38, 41, 42, 52, 72

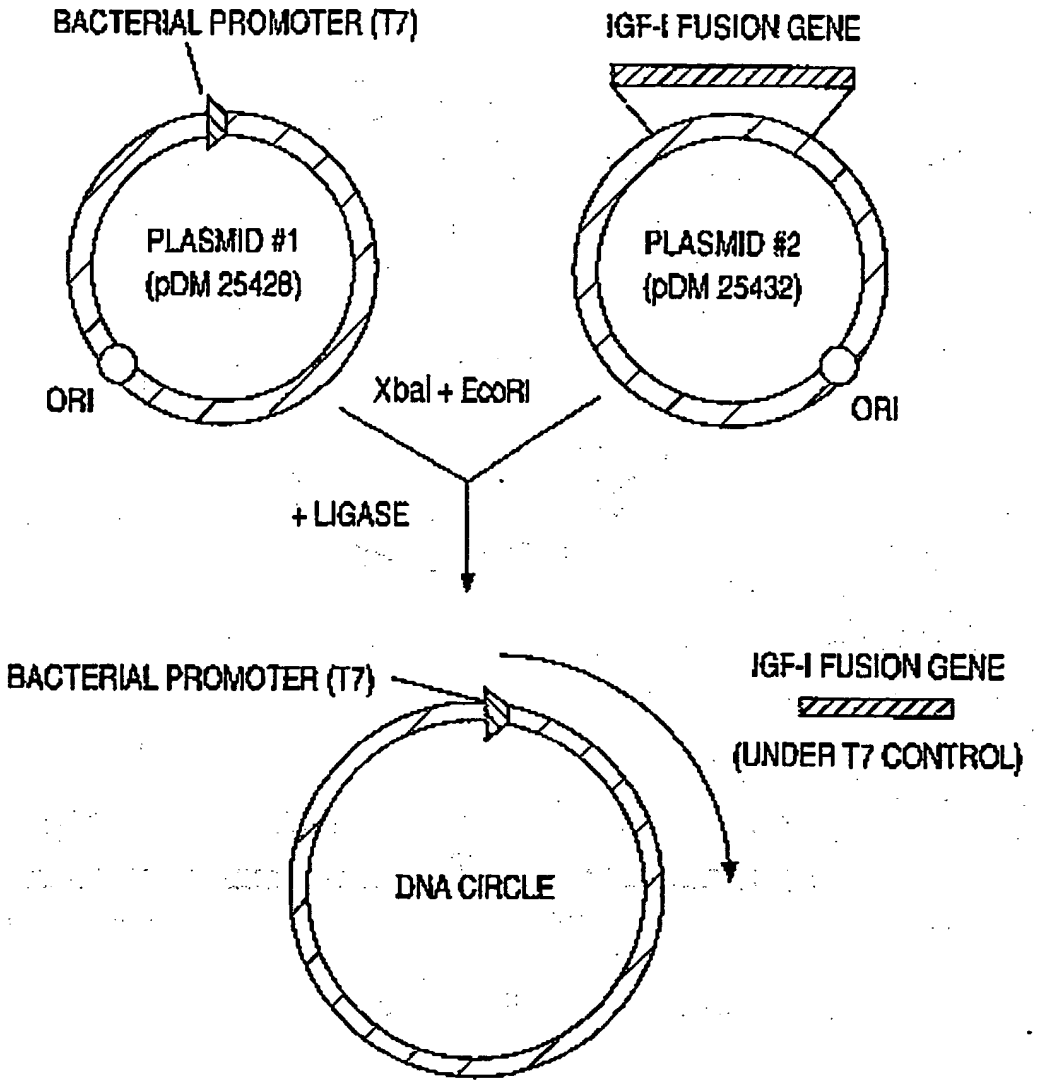


FIG. 1

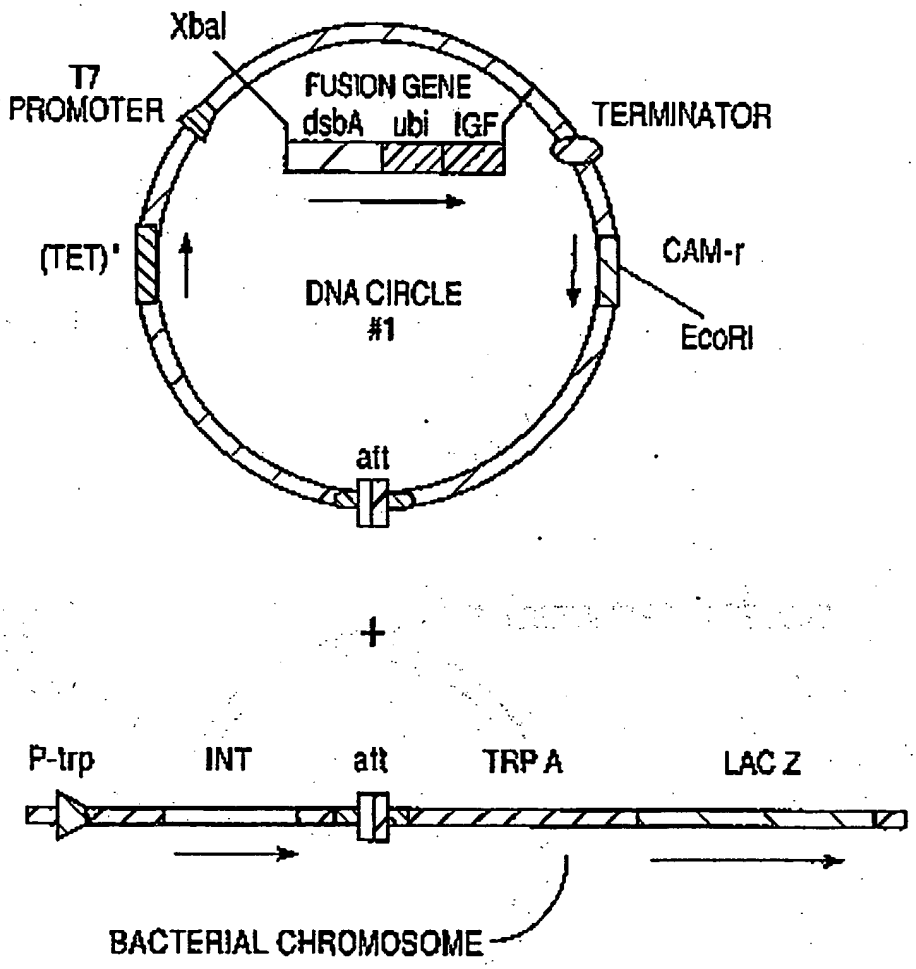

XbaI
T7
PROMOTER
FUSION GENE
dsbA ubi IGF
TERMINATOR
(TET)'
CAM-r
DNA CIRCLE
#1
EcoRI
att
+
P-trp
INT
att
TRP A
LAC Z
BACTERIAL CHROMOSOME

FIG. 2

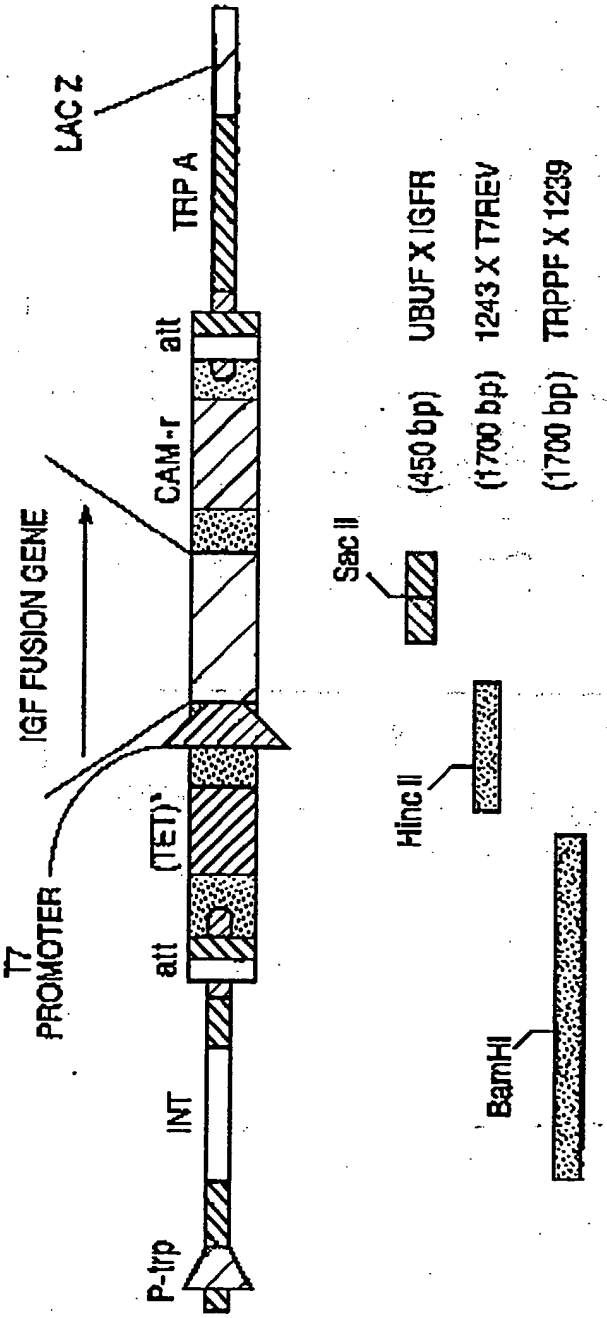


FIG. 3

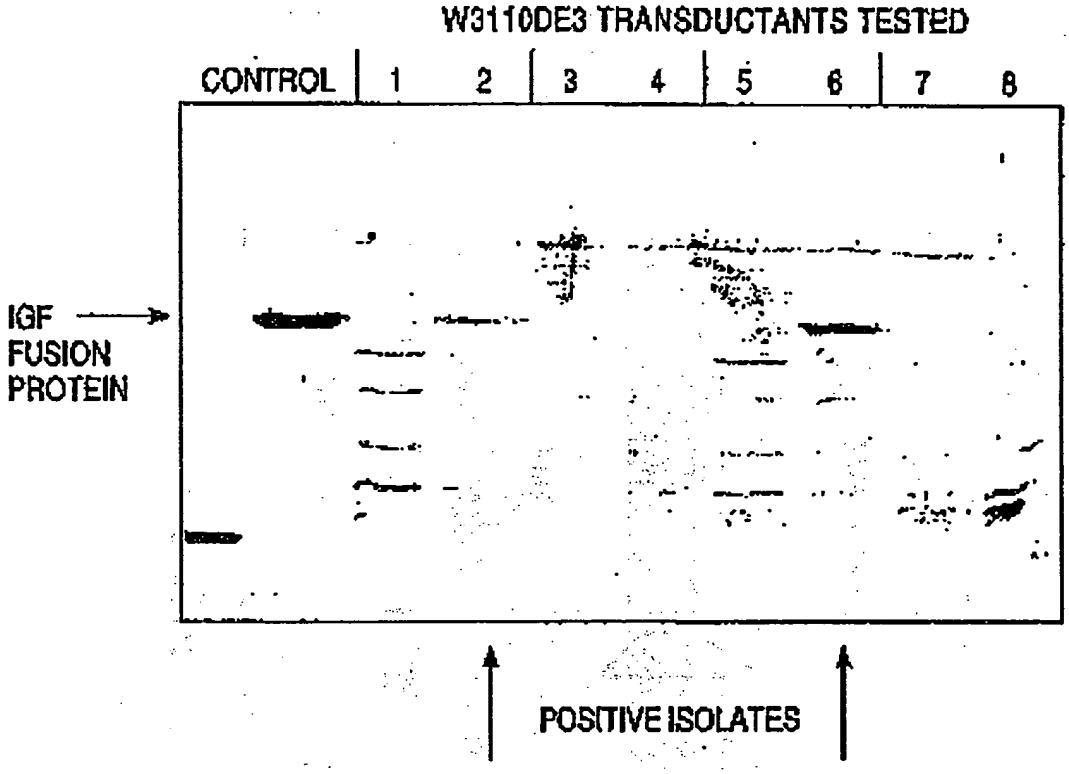

W3110DE3 TRANSDUCTANTS TESTED
CONTROL
1
2
3
4
5
6
7
8
IGF
FUSION
PROTEIN
POSITIVE ISOLATES

FIG. 4

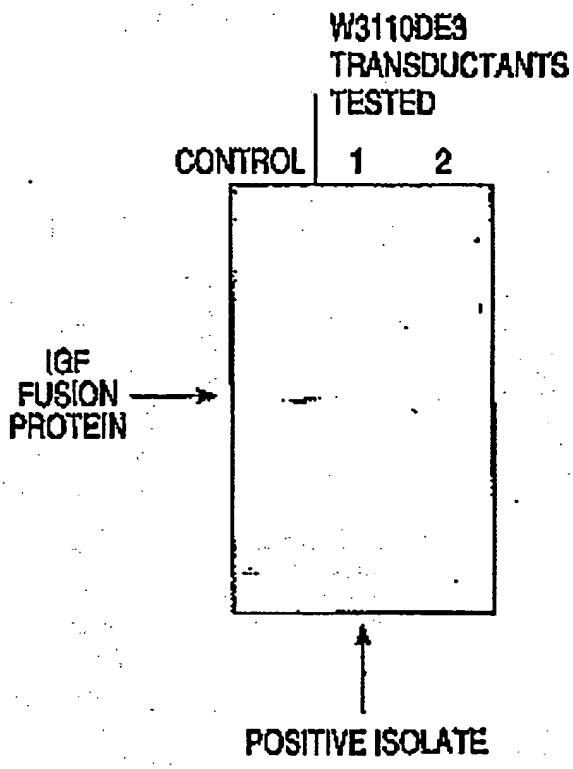

W3110DE3
TRANSDUCTANTS
TESTED
CONTROL
1
2
IGF
FUSION
PROTEIN
POSITIVE ISOLATE

FIG. 5

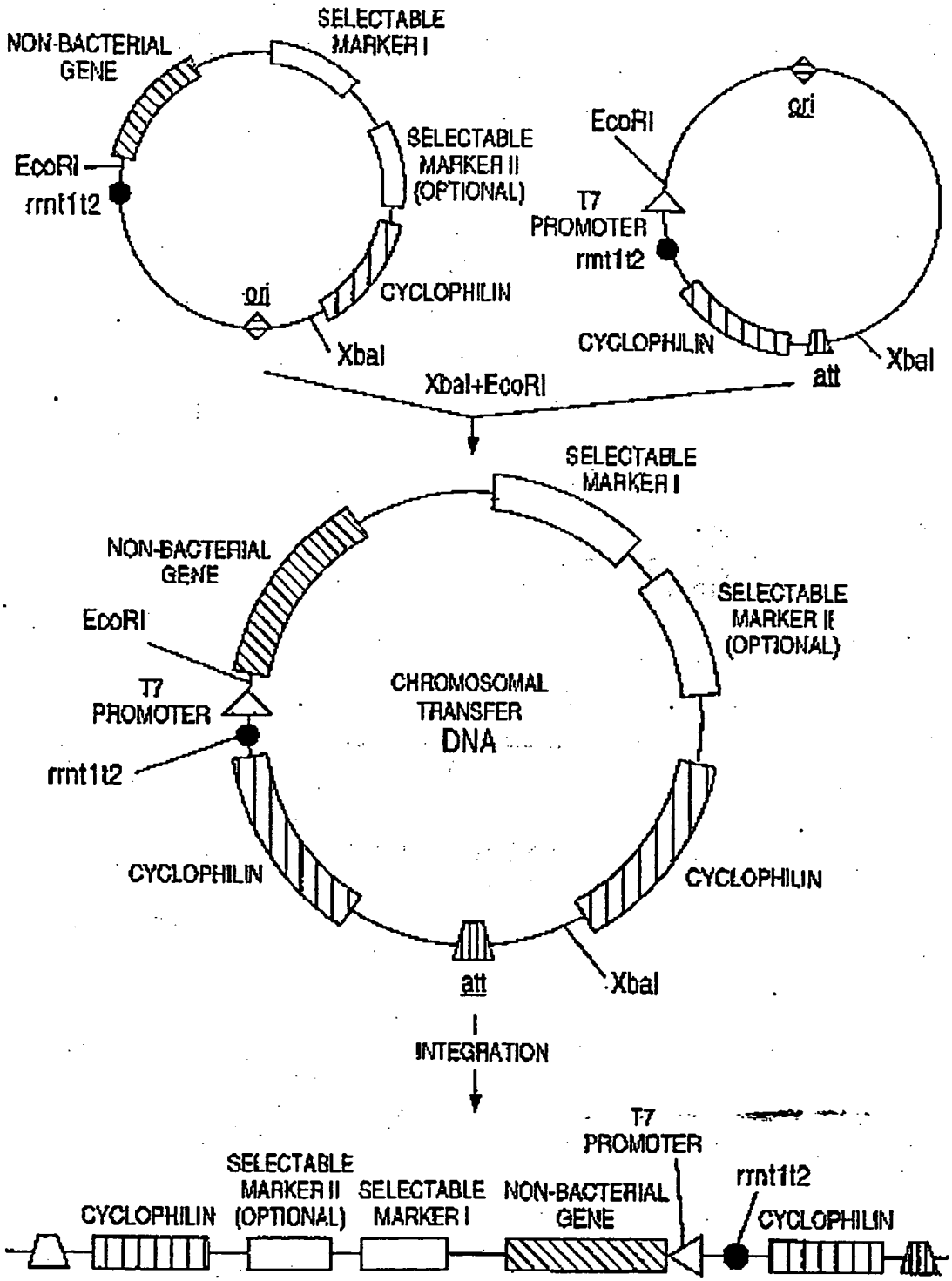

SELECTABLE MARKER I
NON-BACTERIAL GENE
SELECTABLE MARKER II (OPTIONAL)
EcoRI
rrnt1t2
ori
CYCLOPHILIN
XbaI
EcoRI
ori
T7 PROMOTER
rrnt1t2
CYCLOPHILIN
XbaI
att
XbaI+EcoRI
SELECTABLE MARKER I
NON-BACTERIAL GENE
SELECTABLE MARKER II (OPTIONAL)
EcoRI
T7 PROMOTER
rrnt1t2
CHROMOSOMAL TRANSFER DNA
CYCLOPHILIN
CYCLOPHILIN
att
XbaI
INTEGRATION
T7 PROMOTER
SELECTABLE MARKER II (OPTIONAL)
SELECTABLE MARKER I
NON-BACTERIAL GENE
rrnt1t2
CYCLOPHILIN
CYCLOPHILIN

FIG. 6

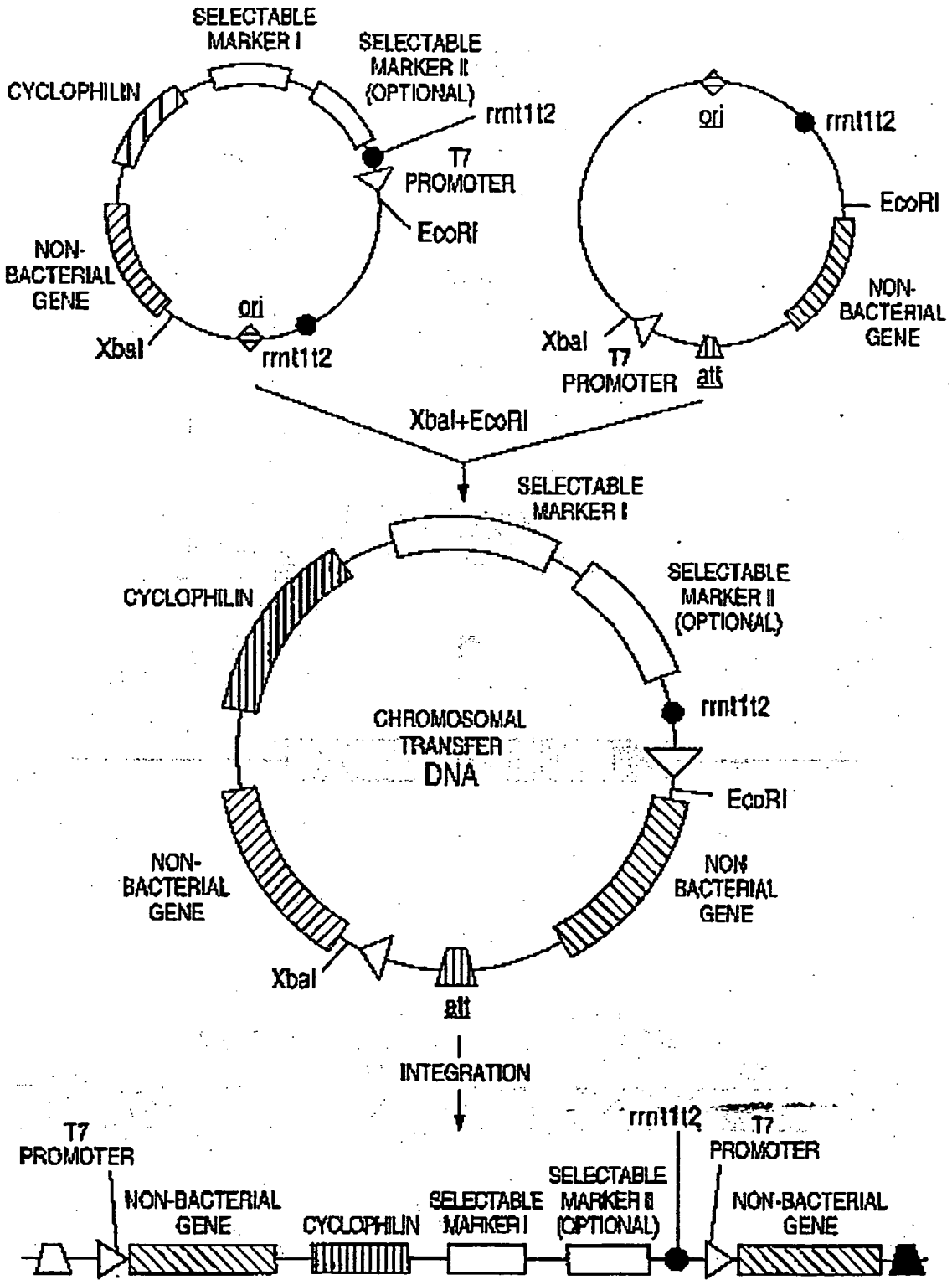

SELECTABLE MARKER I
SELECTABLE MARKER II (OPTIONAL)
CYCLOPHILIN
rrnt1t2
T7 PROMOTER
EcoRI
NON-BACTERIAL GENE
ori
XbaI
rrnt1t2
ori
rrnt1t2
EcoRI
NON-BACTERIAL GENE
XbaI
T7 PROMOTER
att
XbaI+EcoRI
SELECTABLE MARKER I
SELECTABLE MARKER II (OPTIONAL)
CYCLOPHILIN
CHROMOSOMAL TRANSFER DNA
rrnt1t2
EcoRI
NON-BACTERIAL GENE
NON-BACTERIAL GENE
XbaI
att
INTEGRATION
T7 PROMOTER
NON-BACTERIAL GENE
CYCLOPHILIN
SELECTABLE MARKER I
SELECTABLE MARKER II (OPTIONAL)
rrnt1t2
T7 PROMOTER
NON-BACTERIAL GENE

FIG. 7

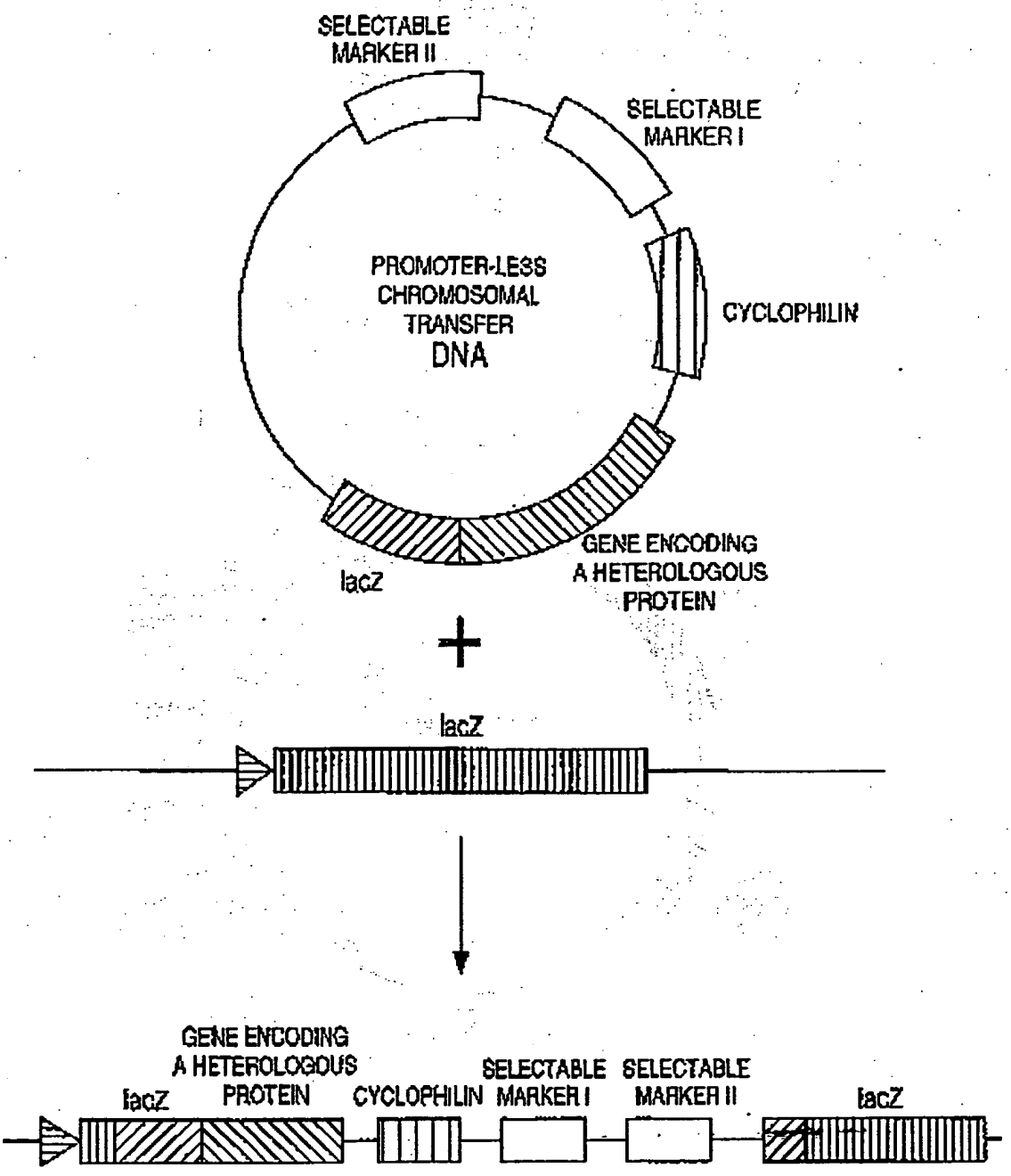

SELECTABLE MARKER II
SELECTABLE MARKER I
PROMOTER-LESS CHROMOSOMAL TRANSFER DNA
CYCLOPHILIN
lacZ
GENE ENCODING A HETEROLOGOUS PROTEIN
lacZ
GENE ENCODING A HETEROLOGOUS PROTEIN
lacZ
CYCLOPHILIN
SELECTABLE MARKER I
SELECTABLE MARKER II
lacZ

FIG. 8

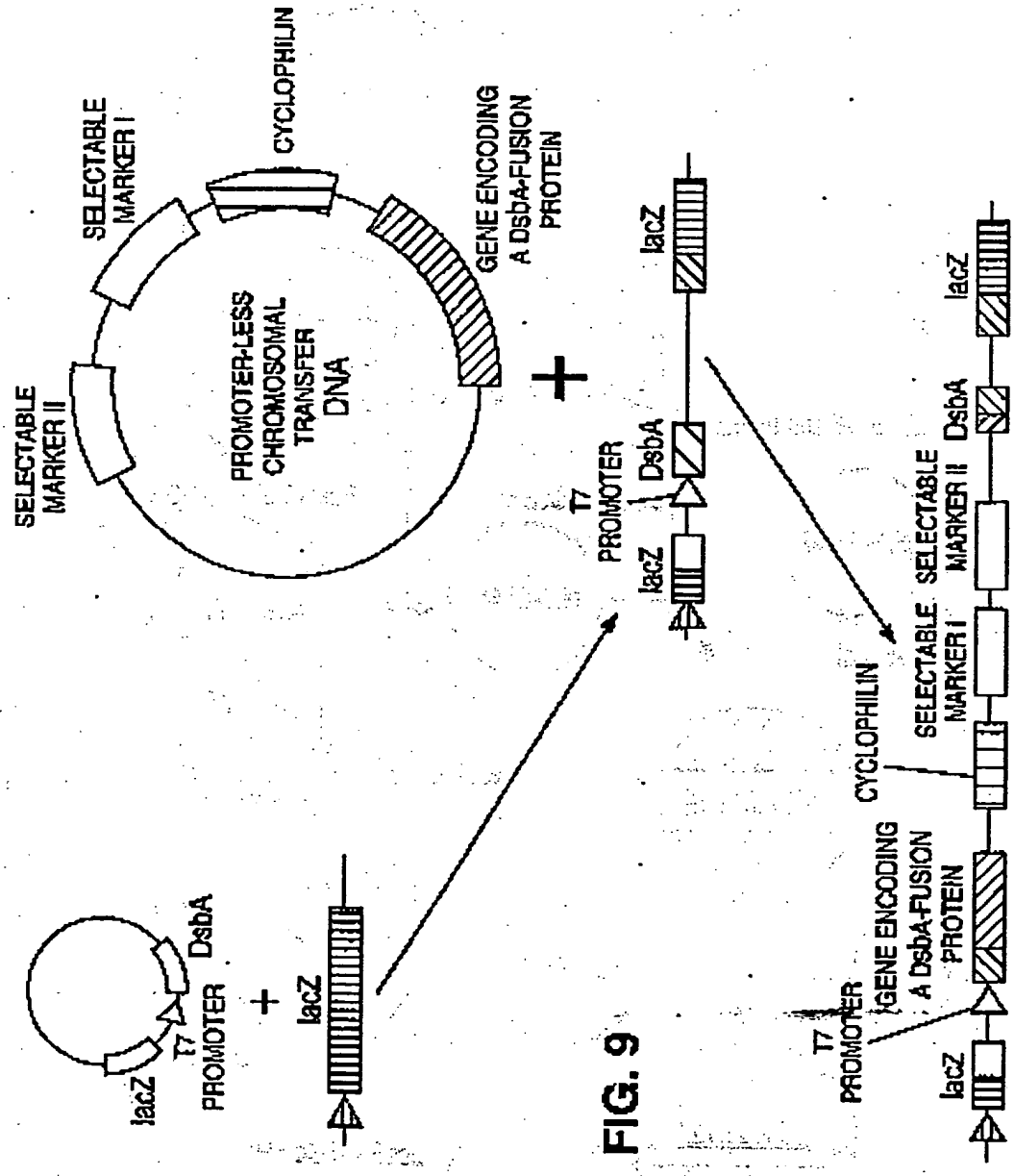


FIG. 9

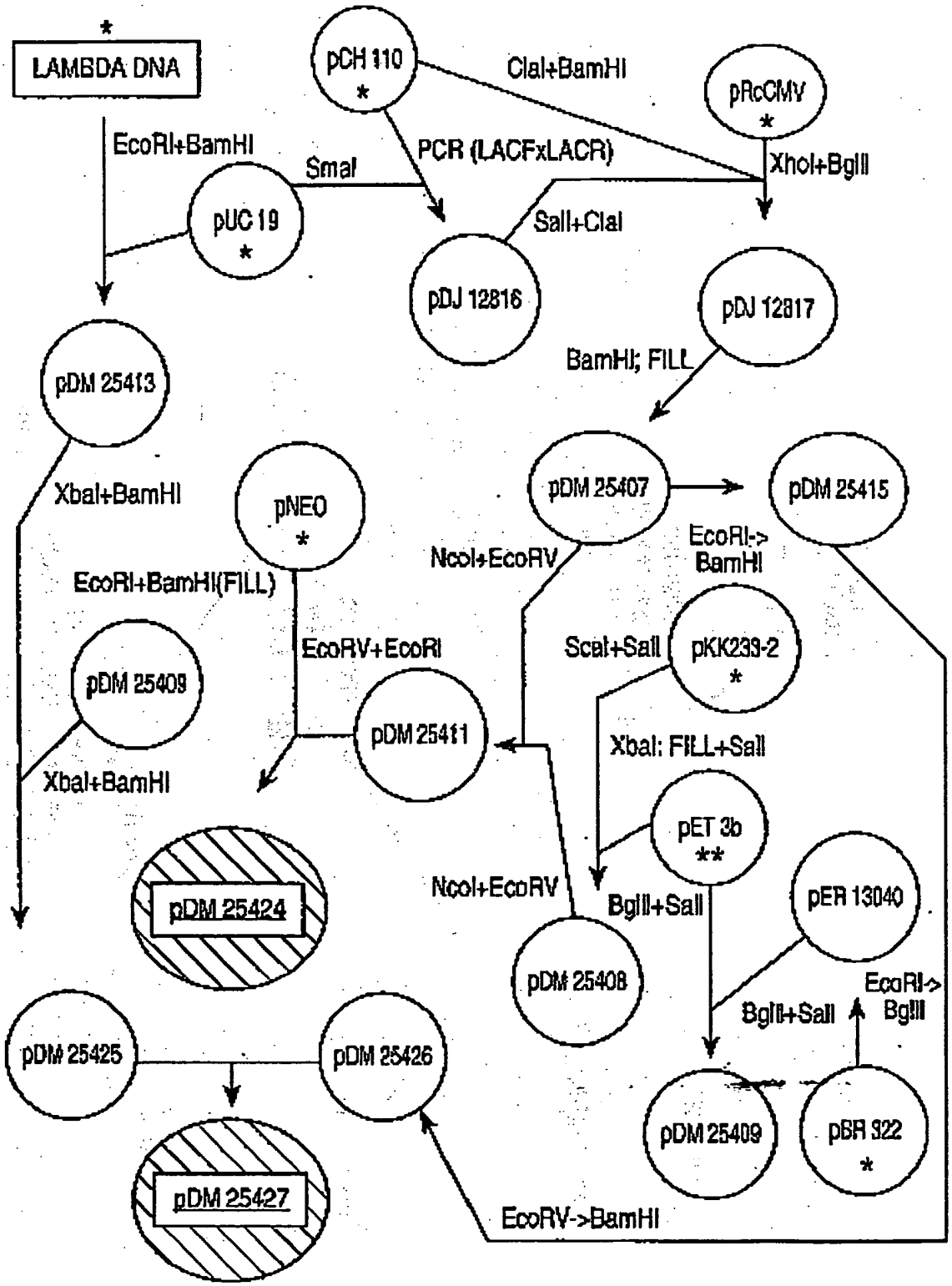


FIG. 10

RECTIFIED SHEET (RULE 91)

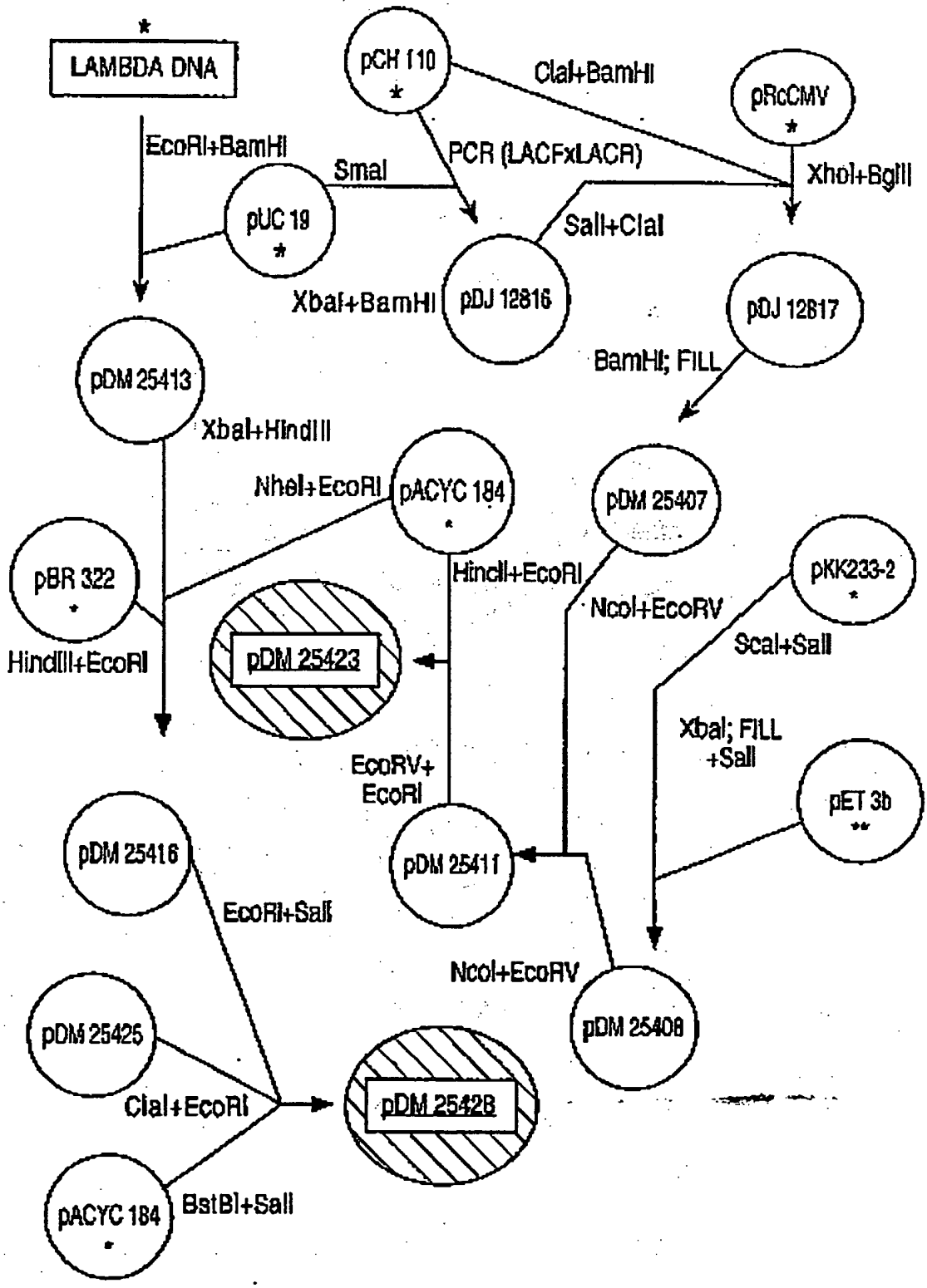


FIG. 11

RECTIFIED SHEET (RULE 91)

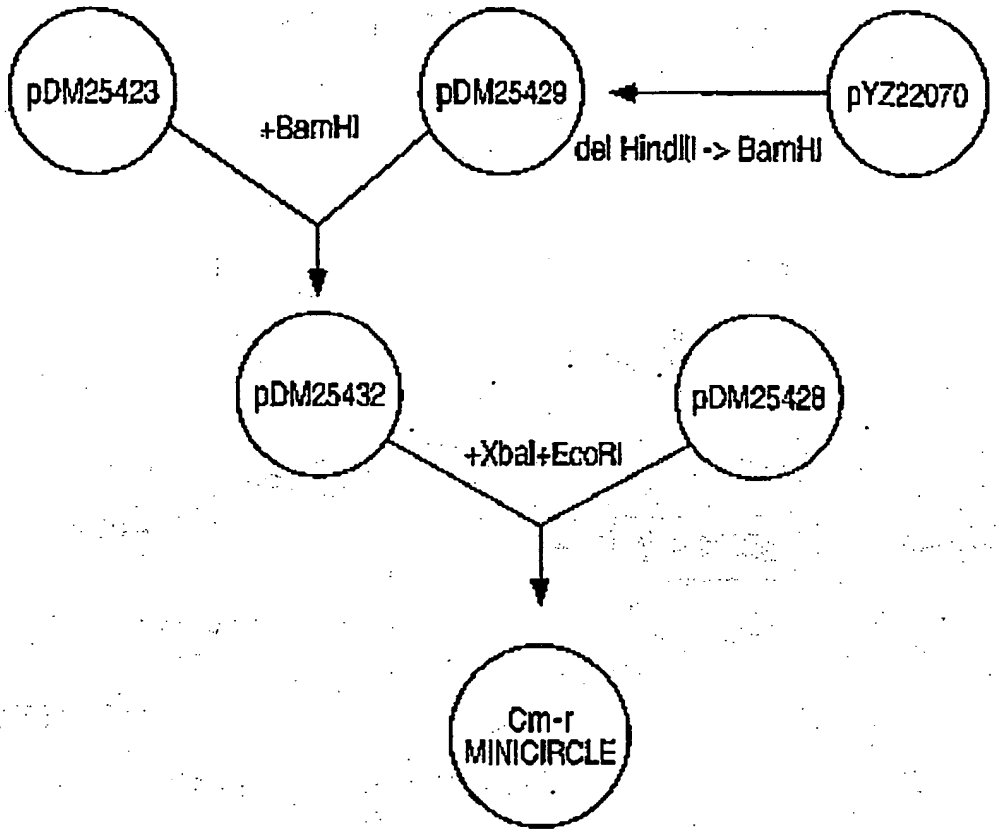

pDM25423
+BamHI
pDM25429
del HindIII -> BamHI
pYZ22070
pDM25432
+XbaI+EcoRI
pDM25428
Cm-r
MINICIRCLE

FIG. 12

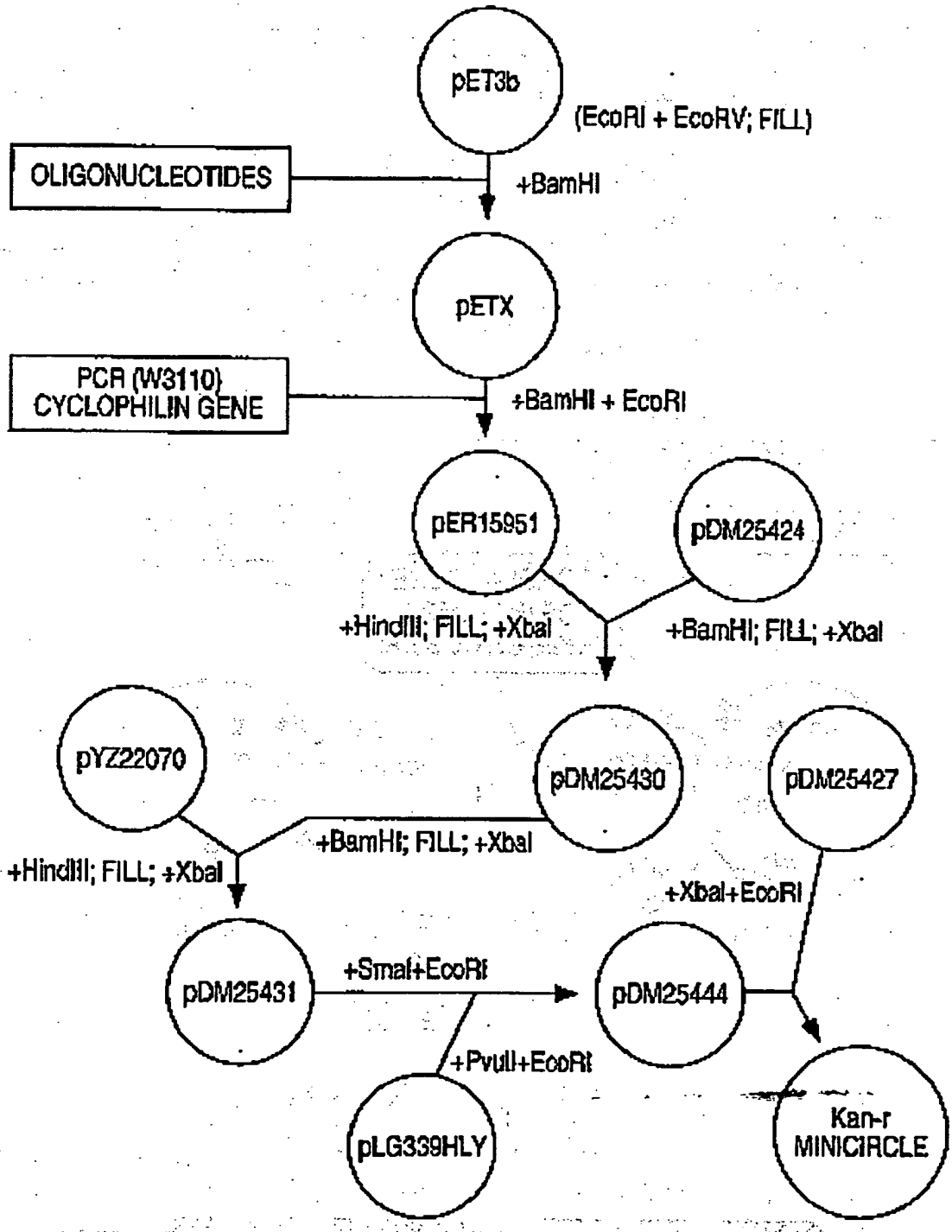


FIG. 13

RECTIFIED SHEET (RULE 91)

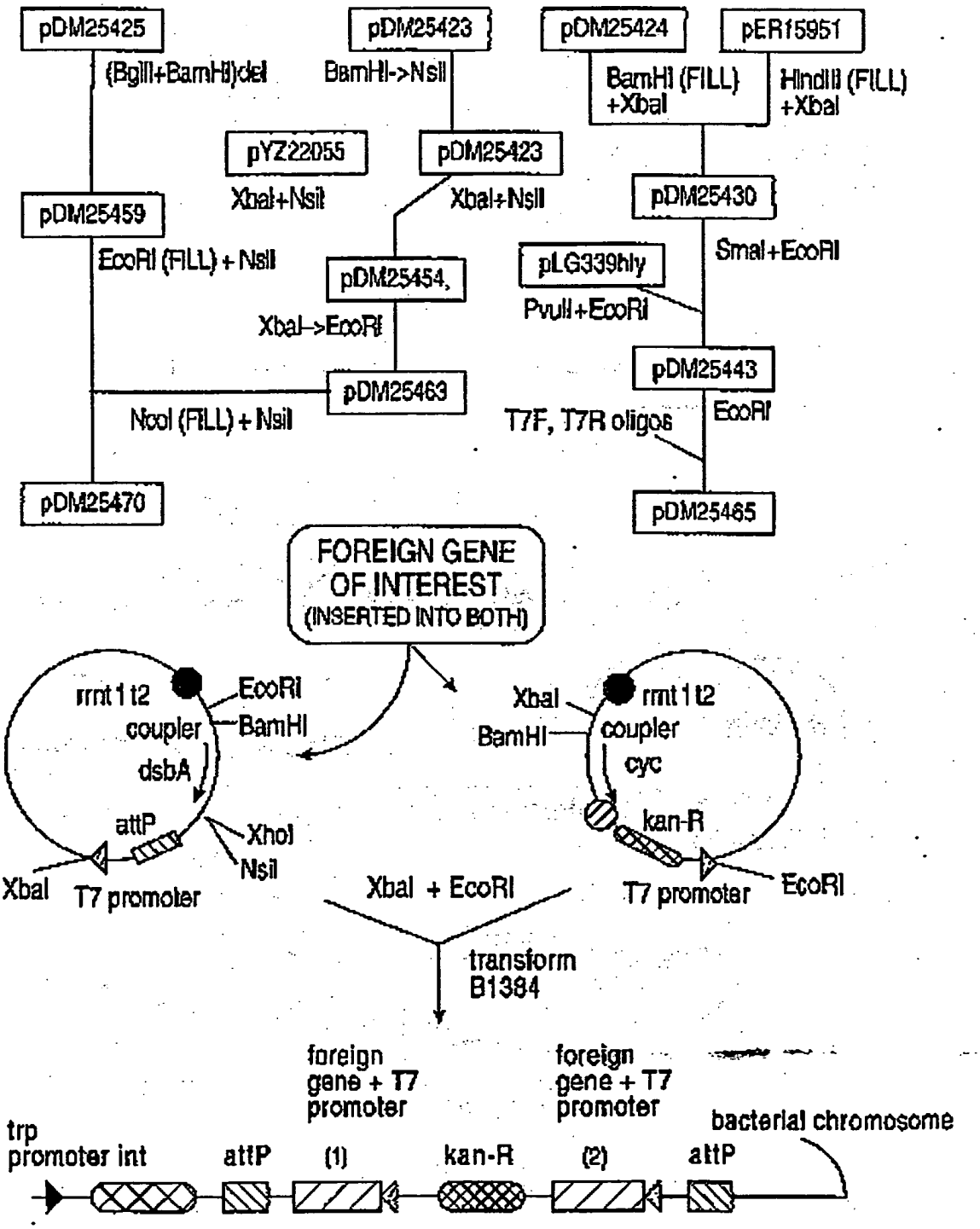

pDM25425
(BglII+BamHI)del
pDM25423
BamHI->NsiI
pDM25424
BamHI (FILL) +XbaI
pER15951
HindIII (FILL) +XbaI
pYZ22055
XbaI+NsiI
pDM25423
XbaI+NsiI
pDM25430
pDM25459
EcoRI (FILL) + NsiI
pDM25454,
XbaI->EcoRI
pLG339hly
PvuII+EcoRI
SmaI+EcoRI
pDM25443
EcoRI
pDM25469
NcoI (FILL) + NsiI
T7F, T7R oligos
pDM25470
pDM25465
FOREIGN GENE OF INTEREST (INSERTED INTO BOTH)
rrnt1t2
coupler
dsbA
EcoRI
BamHI
attP
XhoI
NsiI
XbaI
T7 promoter
XbaI
BamHI
rrnt1t2
coupler
cyc
kan-R
T7 promoter
EcoRI
XbaI + EcoRI
transform B1384
foreign gene + T7 promoter
foreign gene + T7 promoter
bacterial chromosome
trp promoter int
attP
(1)
kan-R
(2)
attP

FIG. 14

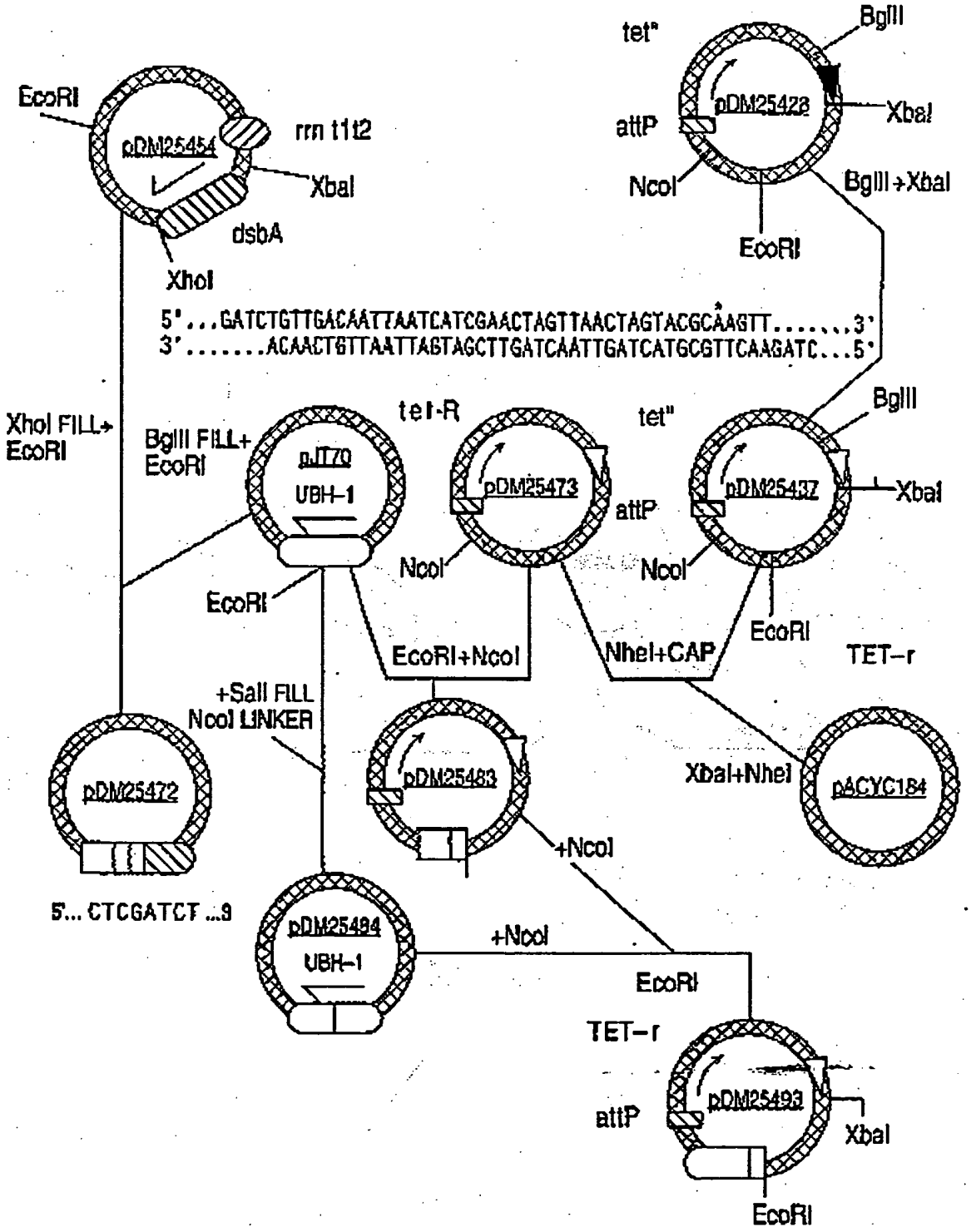

EcoRI
pDM25454
rrn t1t2
XbaI
dsbA
XhoI
tet"
BglII
pDM25428
XbaI
attP
NcoI
BglII+XbaI
EcoRI
5'...GATCTGTTGACAATTAATCATCGAACTAGTTAACTAGTACGCAAGTT.....3'
3'.....ACAACTGTTAATTAGTAGCTTGATCAATTGATCATGCGTTCAAGATC...5'
XhoI FILL+
EcoRI
BglII FILL+
EcoRI
pJT70
UBH-1
tet-R
pDM25473
attP
tet"
BglII
pDM25427
XbaI
NcoI
NcoI
EcoRI
EcoRI+NcoI
NheI+CAP
EcoRI
TET-r
+SalI FILL
NcoI LINKER
pDM25472
pDM25483
XbaI+NheI
pACYC184
+NcoI
5'... CTCGATCT ...3
pDM25484
UBH-1
+NcoI
EcoRI
TET-r
attP
pDM25493
XbaI
EcoRI

FIG. 15

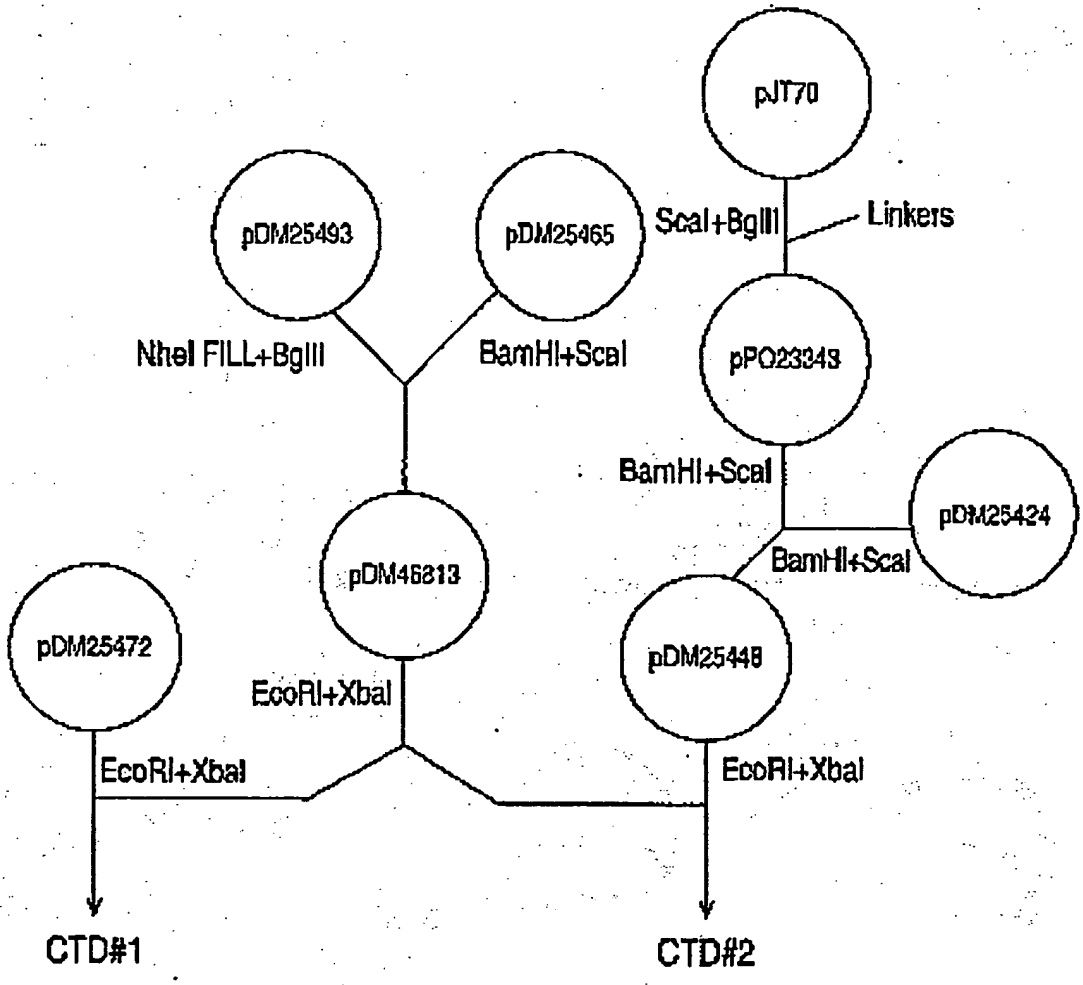

pJT70
pDM25493
pDM25465
ScaI+BglII
Linkers
NheI FILL+BglII
BamHI+ScaI
pPO23343
BamHI+ScaI
pDM25424
pDM46813
BamHI+ScaI
pDM25472
pDM25448
EcoRI+XbaI
EcoRI+XbaI
EcoRI+XbaI
CTD#1
CTD#2

FIG. 16

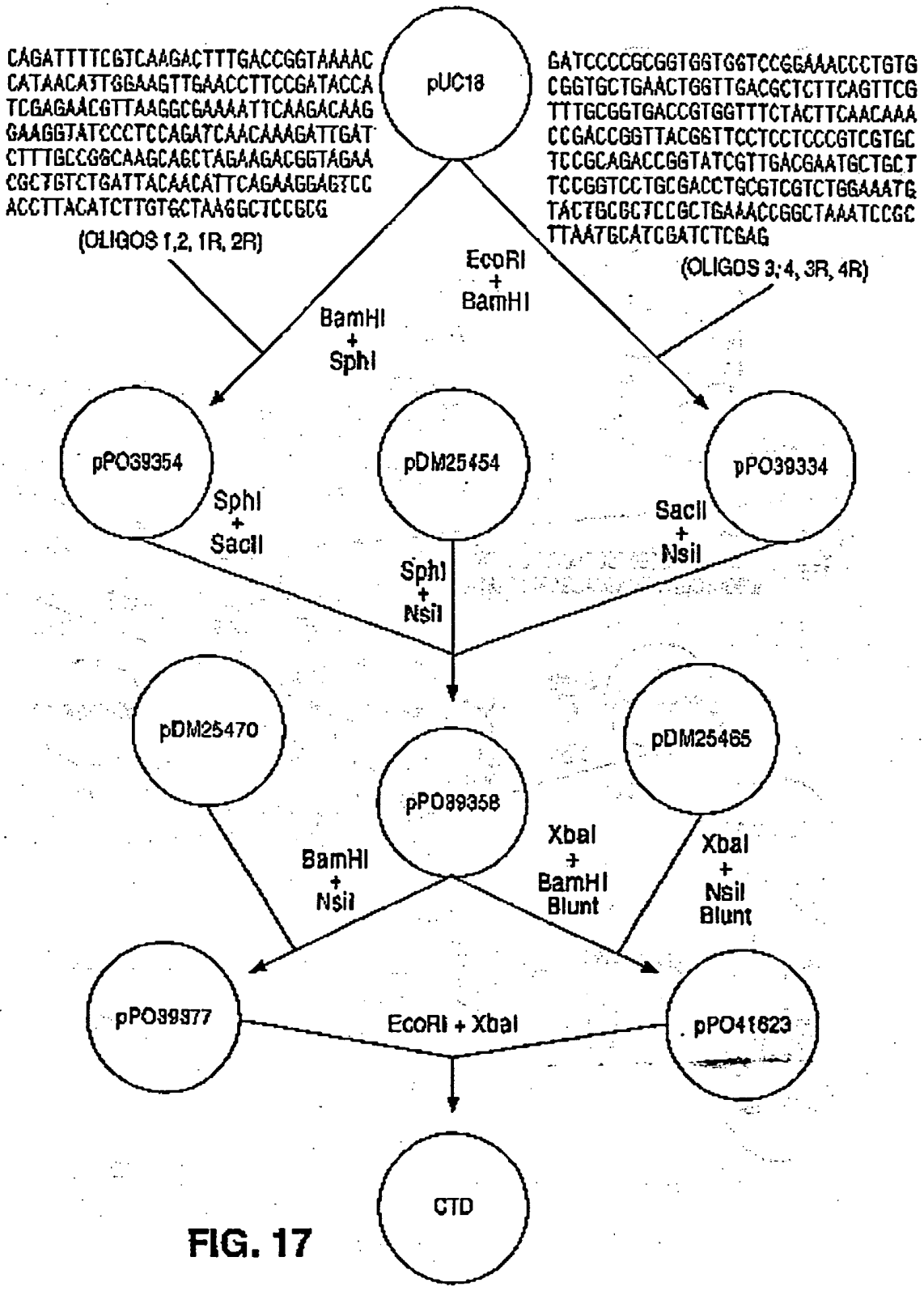

CAGATTTTCGTCAAGACTTTGACCGGTAAAAC
CATAACATTGGAAGTTGAACCTTCCGATACCA
TCGAGAACGTTAAGGCGAAAATTCAAGACAAG
GAAGGTATCCCTCCAGATCAACAAAGATTGAT
CTTTGCCGGCAAGCAGCTAGAAGACGGTAGAA
CGCTGTCTGATTACAACATTCAGAAGGAGTCC
ACCTTACATCTTGTGCTAAGGCTCCGCG
(OLIGOS 1,2, 1R, 2R)
pUC18
GATCCCCGCGGTGGTGGTCCGGAAACCCTGTG
CGGTGCTGAACTGGTTGACGCTCTTCAGTTCG
TTTGCGGTGACCGTGGTTTCTACTTCAACAAA
CCGACCGGTTACGGTTCCTCCTCCCGTCGTGC
TCCGCAGACCGGTATCGTTGACGAATGCTGCT
TCCGGTCCTGCGACCTGCGTCGTCTGGAAATG
TACTGCGCTCCGCTGAAACCGGCTAAATCCGC
TTAATGCATCGATCTCGAG
(OLIGOS 3, 4, 3R, 4R)
BamHI + SphI
EcoRI + BamHI
pPO39354
pDM25454
pPO39334
SphI + SacII
SacII + NsiI
SphI + NsiI
pDM25470
pPO39358
pDM25465
BamHI + NsiI
XbaI + BamHI Blunt
XbaI + NsiI Blunt
pPO39977
EcoRI + XbaI
pPO41623
CTD

FIG. 17

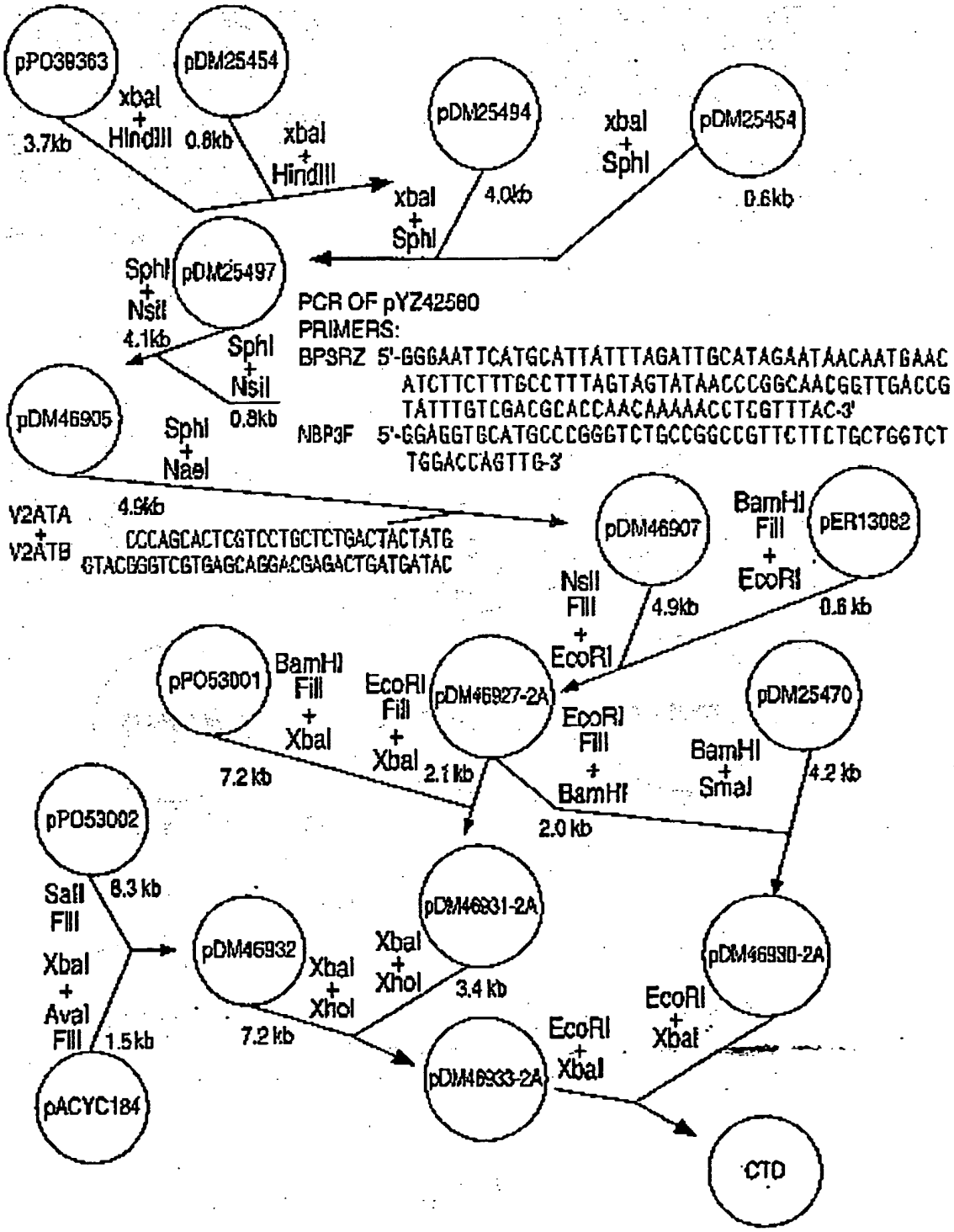

pPO39363
pDM25454
xbal + HindIII
3.7kb
0.8kb
xbal + HindIII
pDM25494
4.0kb
xbal + SphI
pDM25454
0.6kb
SphI + NsiI
pDM25497
4.1kb
SphI + NsiI
0.8kb
PCR OF pYZ42580
PRIMERS:
BP3RZ 5'-GGGAATTCATGCATTATTTAGATTGCATAGAATAACAATGAAC
ATCTTCTTTGCCTTTAGTAGTATAACCCGGCAACGGTTGACCG
TATTTGTCGACGCACCAACAAAAACCTCGTTTAC-3'
NBP3F 5'-GGAGGTGCATGCCCGGGTCTGCCGGCCGTTCTTCTGCTGGTCT
TGGACCAGTTG-3'
pDM46905
SphI + NaeI
4.9kb
V2ATA + V2ATB
CCCAGCACTCGTCCTGCTCTGACTACTATG
GTACGGGTCGTGAGCAGGACGAGACTGATGATAC
pDM46907
NsiI FIII + EcoRI
4.9kb
BamHI FIII + EcoRI
pER13082
0.6 kb
pPO53001
BamHI FIII + XbaI
7.2 kb
EcoRI FIII + XbaI
pDM46927-2A
2.1 kb
EcoRI FIII + BamHI
2.0 kb
pDM25470
BamHI + SmaI
4.2 kb
pPO53002
SalI FIII
8.3 kb
XbaI + AvaI FIII
1.5 kb
pACYC184
pDM46932
XbaI + XhoI
7.2 kb
pDM46931-2A
XbaI + XhoI
3.4 kb
pDM46933-2A
EcoRI + XbaI
pDM46930-2A
EcoRI + XbaI
CTD

FIG. 18

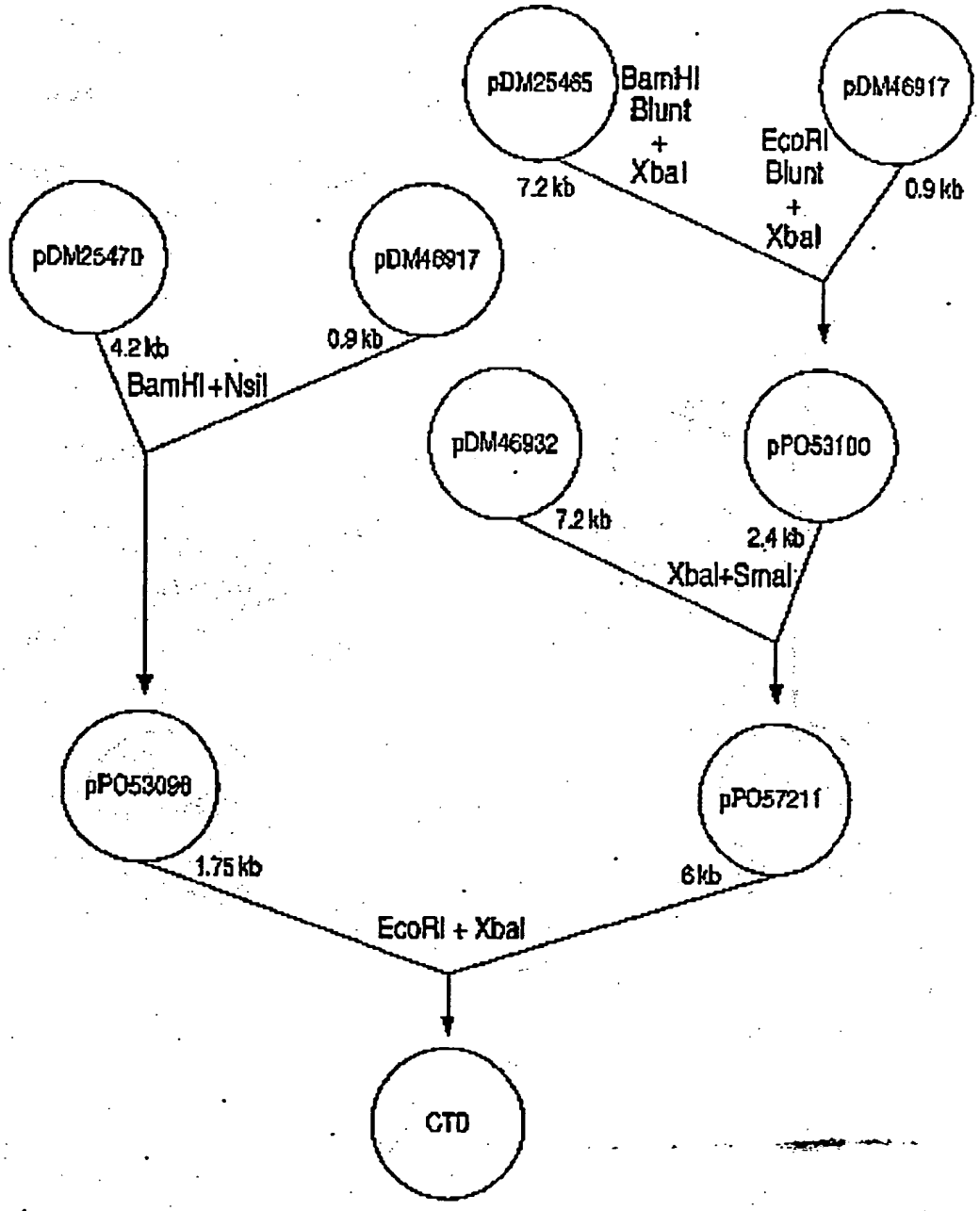


FIG. 19

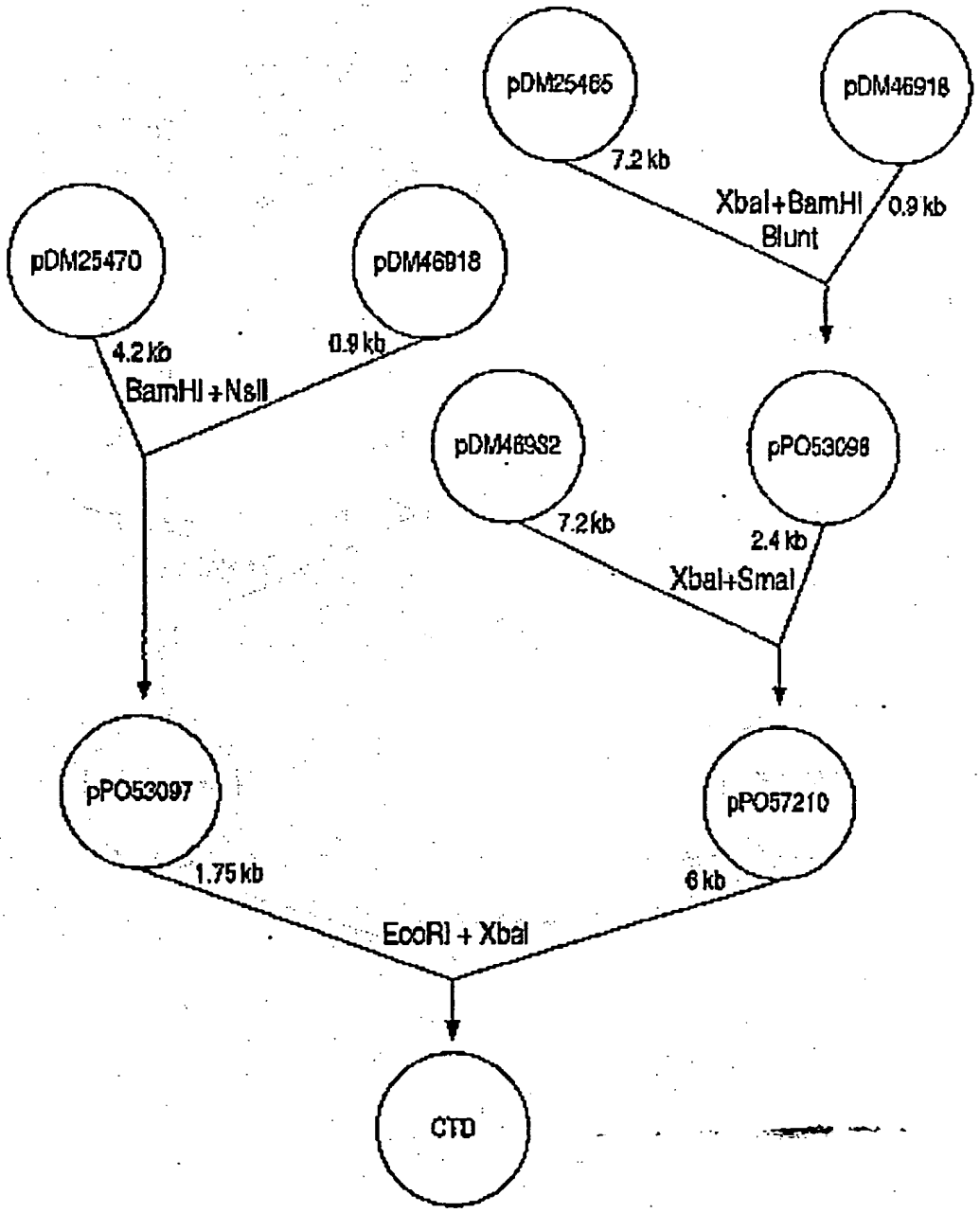

pDM25465
pDM46918
7.2 kb
XbaI+BamHI
Blunt
0.9 kb
pDM25470
pDM46918
4.2 kb
BamHI + NsiI
0.9 kb
pDM46952
pPO53098
7.2kb
2.4 kb
XbaI+SmaI
pPO53097
pPO57210
1.75 kb
6 kb
EcoRI + XbaI
CTD

FIG. 20

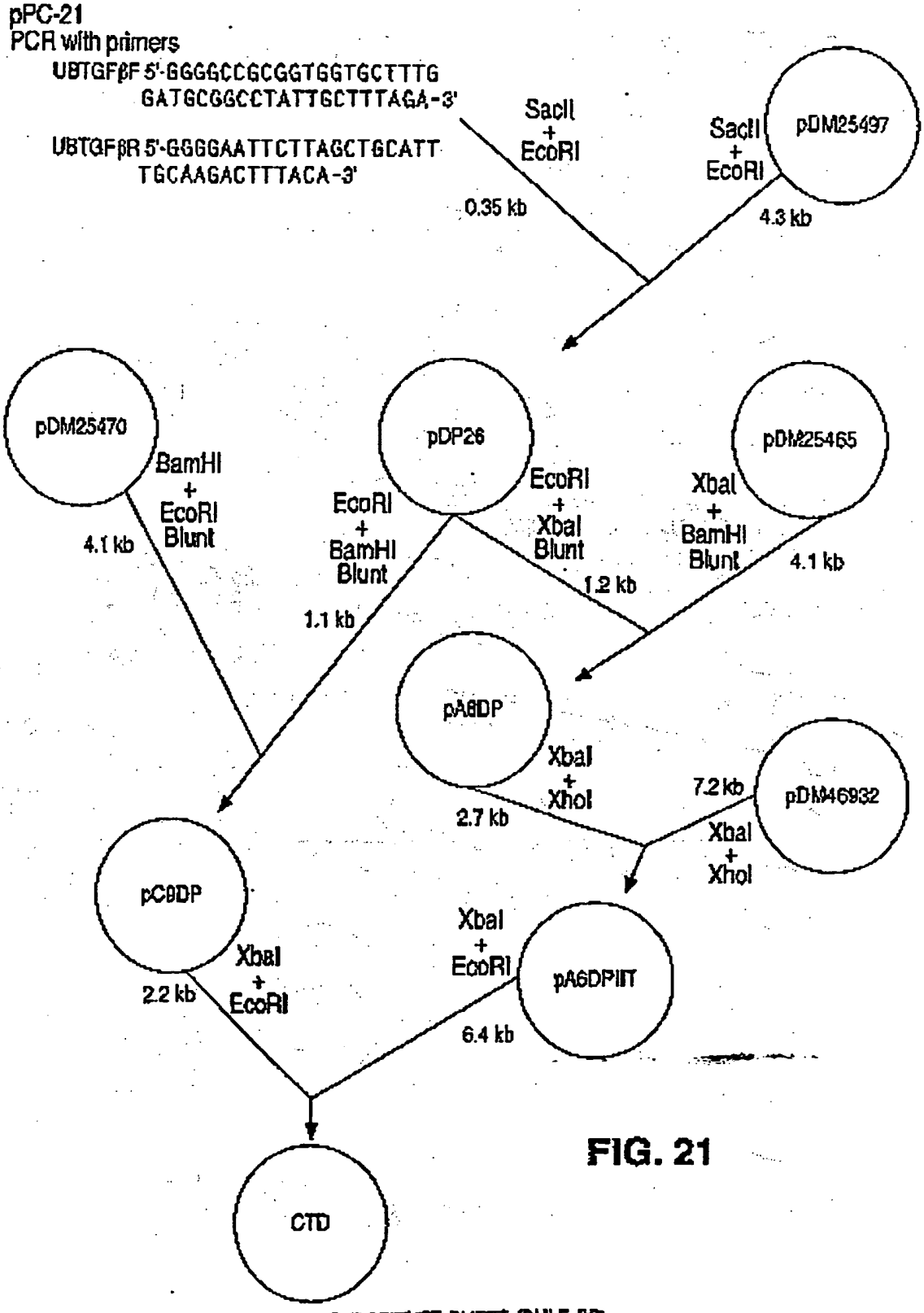


FIG. 21

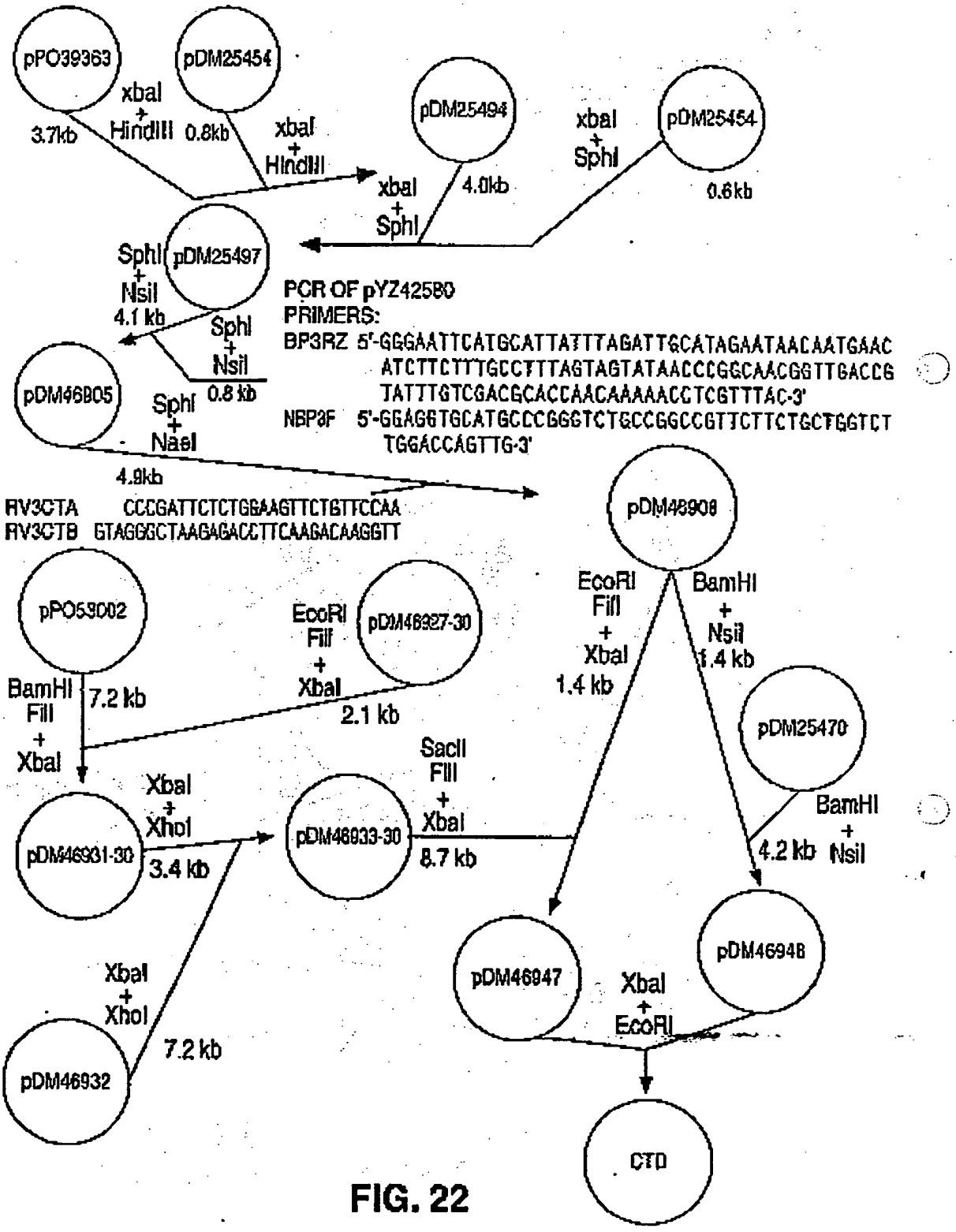

pPO39363
pDM25454
xbal + HindIII
3.7kb
0.8kb
xbal + HindIII
pDM25494
4.0kb
xbal + SphI
pDM25454
0.6kb
xbal + SphI
SphI + NsiI
pDM25497
4.1 kb
SphI + NsiI
0.8 kb
pDM46805
SphI + NaeI
4.9kb
PCR OF pYZ42580
PRIMERS:
BP3RZ 5'-GGGAATTCATGCATTATTTAGATTGCATAGAATAACAATGAAC
ATCTTCTTTGCCTTTAGTAGTATAACCCGGCAACGGTTGACCG
TATTTGTCGACGCACCAACAAAAACCTCGTTTAC-3'
NBP3F 5'-GGAGGTGCATGCCCGGGTCTGCCGGCCGTTCTTCTGCTGGTCT
TGGACCAGTTG-3'
RV3CTA CCCGATTCTCTGGAAGTTCTGTTCCAA
RV3CTB GTAGGGCTAAGAGACCTTCAAGACAAGGTT
pDM46908
pPO59002
BamHI FiII + XbaI
7.2 kb
EcoRI FiII + XbaI
pDM46927-30
2.1 kb
EcoRI FiII + XbaI
1.4 kb
BamHI + NsiI
1.4 kb
pDM25470
BamHI + NsiI
4.2 kb
XbaI + XhoI
pDM46931-30
3.4 kb
pDM46933-30
SacII FiII + XbaI
8.7 kb
pDM46947
pDM46948
XbaI + EcoRI
XbaI + XhoI
pDM46932
7.2 kb
CTD

FIG. 22

FIG. 23

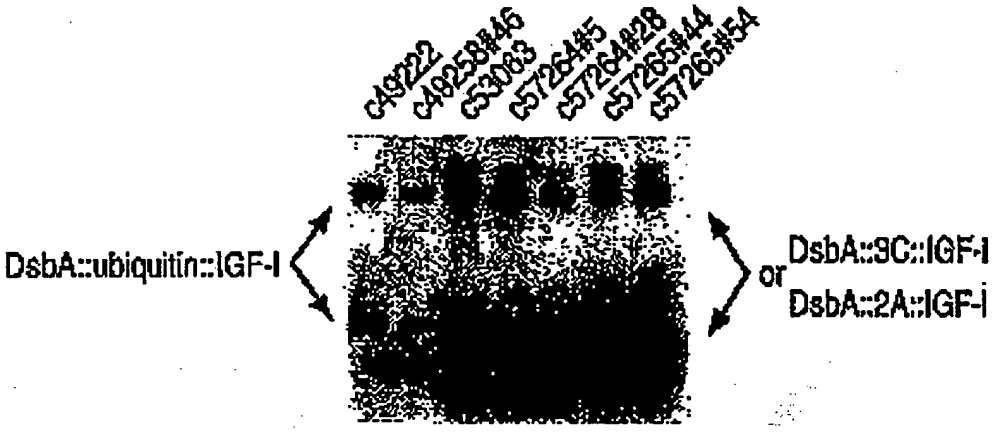

c49f22
c49f258#46
c53063
c57264#5
c57264#28
c57265#44
c57265#54
DsbA::ubiquitin::IGF-I
or
DsbA::3C::IGF-I
DsbA::2A::IGF-I

FIG. 24

FIG. 25A

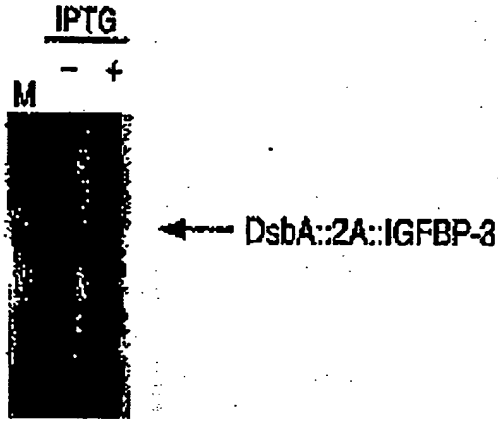
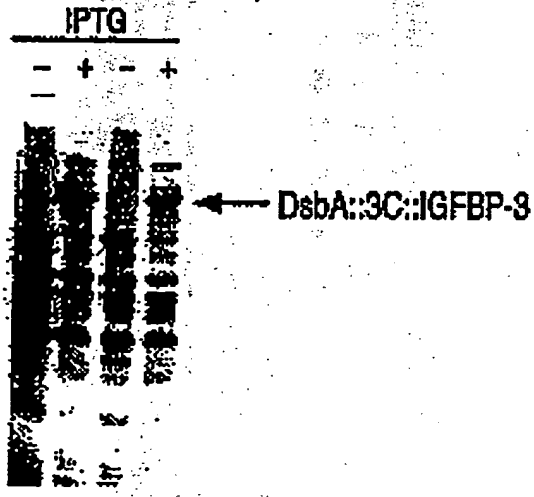


FIG. 25B



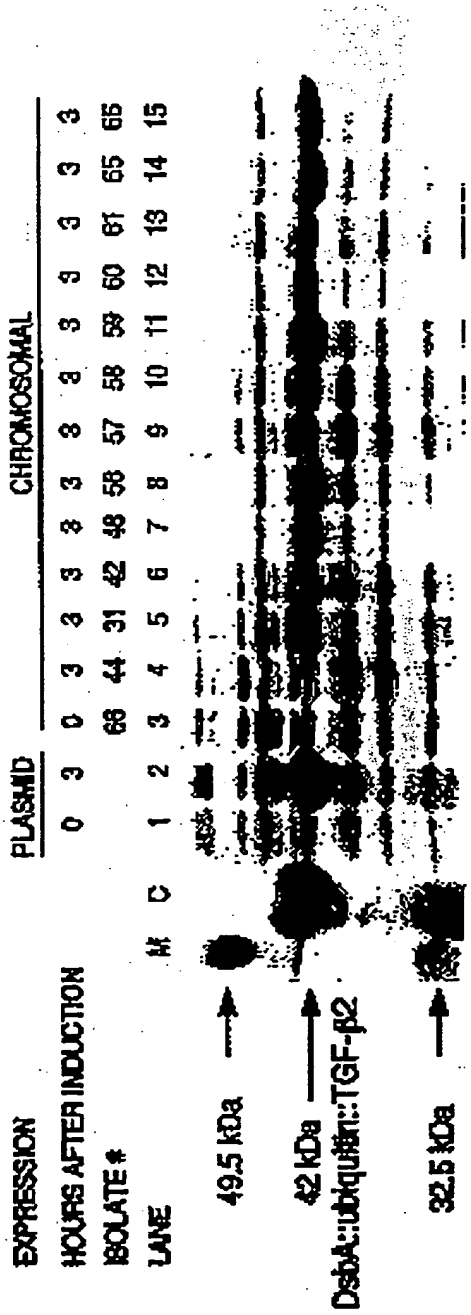

EXPRESSION
PLASMID
CHROMOSOMAL
HOURS AFTER INDUCTION
0 3 0 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3
ISOLATE #
68 44 31 42 48 58 57 58 59 60 61 65 65
LANE
M C 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
49.5 kDa
42 kDa
DsbA::ubiquitin::TGF-β2
32.5 kDa

FIG. 26